

(12)

(21) 2 441 442

(51) Int. Cl.<sup>7</sup>: **A61K 47/48**

(22) 15.03.2002

(85) 26.09.2003

(86) PCT/EP02/02928

(87) WO02/080979

(30) 101 12 825.8 DE 16.03.2001

(71) FRESenius KABI DEUTSCHLAND GMBH,  
Else-Kroner-Strasse 1  
61352, BAD HOMBURG, XX (DE).

FRIE, SVEN (DE).  
JUNGHEINRICH, CORNELIUS (DE).  
SCHARPF, ROLAND (DE).  
LUTTERBECK, KATHARINA (DE).  
EICHNER, WOLFRAM (DE).  
SOMMERMEYER, KLAUS (DE).

(72)

(74)

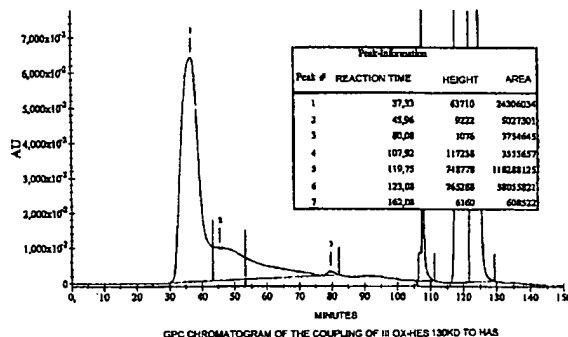
RIDOUT & MAYBEE LLP

(54) CONJUGUES D'AMIDON HYDROXYALKYLE ET D'AGENT ACTIF

(54) CONJUGATE OF HYDROXYALKYL STARCH AND AN ACTIVE AGENT

(57)

The invention relates to compounds, comprising a conjugate of hydroxyalkyl starch (HAS) and an active agent, whereby the hydroxyalkyl starch is either directly covalently bonded to the active agent, or by means of a linker. The invention further relates to methods for the production of a covalent HAS- active agent conjugate, whereby HAS and an active agent are reacted in a reaction medium, characterised in that the reaction medium is water or a mixture of water and an organic solvent, comprising at least 10 wt. % water.





Office de la Propriété  
Intellectuelle  
du Canada

Un organisme  
d'Industrie Canada

Canadian  
Intellectual Property  
Office

An agency of  
Industry Canada

CA 2441442 A1 2002/10/17

(21) **2 441 442**

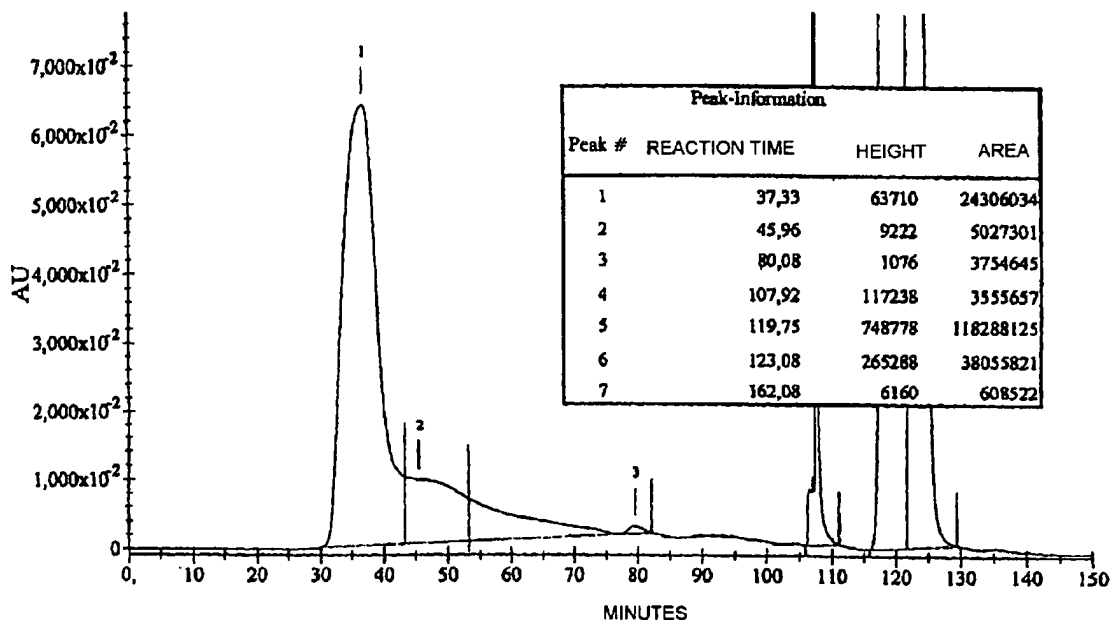
(12) **DEMANDE DE BREVET CANADIEN  
CANADIAN PATENT APPLICATION**

(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2002/03/15  
(87) Date publication PCT/PCT Publication Date: 2002/10/17  
(85) Entrée phase nationale/National Entry: 2003/09/26  
(86) N° demande PCT/PCT Application No.: EP 2002/002928  
(87) N° publication PCT/PCT Publication No.: 2002/080979  
(30) Priorité/Priority: 2001/03/16 (101 12 825.8) DE

(51) Cl.Int.<sup>7</sup>/Int.Cl.<sup>7</sup> A61K 47/48  
(71) Demandeur/Applicant:  
FRESENIUS KABI DEUTSCHLAND GMBH, DE  
(72) Inventeurs/Inventors:  
SOMMERMEYER, KLAUS, DE;  
EICHNER, WOLFRAM, DE;  
FRIE, SVEN, DE;  
JUNGHEINRICH, CORNELIUS, DE;  
SCHARPF, ROLAND, DE;  
LUTTERBECK, KATHARINA, DE  
(74) Agent: RIDOUT & MAYBEE LLP

(54) Titre : CONJUGUES D'AMIDON HYDROXYALKYLE ET D'AGENT ACTIF  
(54) Title: CONJUGATE OF HYDROXYALKYL STARCH AND AN ACTIVE AGENT



GPC CHROMATOGRAM OF THE COUPLING OF III OX-HES 130KD TO HAS

(57) Abrégé/Abstract:

The invention relates to compounds, comprising a conjugate of hydroxyalkyl starch (HAS) and an active agent, whereby the hydroxyalkyl starch is either directly covalently bonded to the active agent, or by means of a linker. The invention further relates to methods for the production of a covalent HAS-active agent conjugate, whereby HAS and an active agent are reacted in a reaction medium, characterised in that the reaction medium is water or a mixture of water and an organic solvent, comprising at least 10 wt. % water.

Canada

<http://opic.gc.ca> · Ottawa-Hull K1A 0C9 · <http://cipo.gc.ca>

OPIC · CIP0 191

OPIC



CIP0

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES  
PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG(19) Weltorganisation für geistiges Eigentum  
Internationales Büro(43) Internationales Veröffentlichungsdatum  
17. Oktober 2002 (17.10.2002)

PCT

(10) Internationale Veröffentlichungsnummer  
WO 02/080979 A3

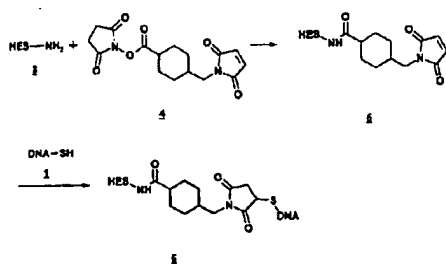
- (51) Internationale Patentklassifikation<sup>7</sup>: A61K 47/48 (72) Erfinder; und  
(21) Internationales Aktenzeichen: PCT/EP02/02928 (75) Erfinder/Anmelder (nur für US): SOMMERMEYER, Klaus [DE/DE]; In der Laubach 26, 61191 Rosbach (DE). EICHNER, Wolfram [DE/DE]; Richard Wagner Str. 12, 35510 Butzbach (DE). FRIE, Sven [DE/DE]; Heuchelheimerstr. 12, 61348 Friedberg (DE). JUNGHEINRICH, Cornelius [DE/DE]; Valkenierstr. 2b, 61350 Bad Nomburg (DE). SCHARPF, Roland [DE/DE]; Vogelshwegstr. 3, 63691 Ranstadt (DE). LUTTERBECK, Katharina [DE/DE]; Am Holzpfortchen 7, 61169 Friedberg (DE).
- (22) Internationales Anmeldedatum: 15. März 2002 (15.03.2002)
- (25) Einreichungssprache: Deutsch
- (26) Veröffentlichungssprache: Deutsch
- (30) Angaben zur Priorität: 101 12 825.8 16. März 2001 (16.03.2001) DE (74) Anwälte: VON MENGES, A. usw.; Uexküll & Stolberg, Beselerstr. 4, 22607 Hamburg (DE).
- (71) Anmelder (für alle Bestimmungsstaaten mit Ausnahme von US): FRESENIUS KABI DEUTSCHLAND GMBH [DE/DE]; Freseniusstrasse, 61159 Friedberg (DE). (81) Bestimmungsstaaten (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR,

[Fortsetzung auf der nächsten Seite]

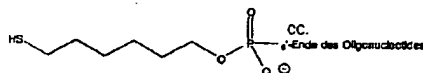
(54) Title: CONJUGATE OF HYDROXYALKYL STARCH AND AN ACTIVE AGENT

(54) Bezeichnung: HYDROXYALKYLSTÄRKE-WIRKSTOFF-KONJUGATE

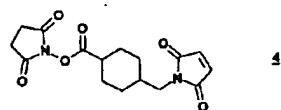
AA schematische Darstellung der Kopplungsreaktion zwischen Amin-HES, SMCC und Thio-DNA:



BB Struktur der Thiomodifikation der DNA



DC Struktur des SMCC



AA... SCHEMATIC REPRESENTATION OF COUPLING REACTION BETWEEN AMIN-HES, SMCC AND THIO-DNA  
BB... STRUCTURE OF THIOMODIFICATION OF DNA  
CC... END OF OLIGONUCLEOTIDES  
DD... STRUCTURE OF THE SMCC

(57) Abstract: The invention relates to compounds, comprising a conjugate of hydroxyalkyl starch (HAS) and an active agent, whereby the hydroxyalkyl starch is either directly covalently bonded to the active agent, or by means of a linker. The invention further relates to methods for the production of a covalent HAS-active agent conjugate, whereby HAS and an active agent are reacted in a reaction medium, characterised in that the reaction medium is water or a mixture of water and an organic solvent, comprising at least 10 wt. % water.

(57) Zusammenfassung: Die vorliegende Erfindung betrifft Verbindungen, die ein Konjugat aus Hydroxyalkylstärke (HAS) und einem Wirkstoff umfassen, wobei die Hydroxyalkylstärke entweder unmittelbar oder über einen Linker kovalent an den Wirkstoff gebunden ist. Die Erfindung betrifft ferner Verfahren zur Herstellung eines kovalenten HAS-Wirkstoff-Konjugates, bei denen man HAS und einen Wirkstoff in einem Reaktionsmedium miteinander umsetzt, dadurch gekennzeichnet, dass das Reaktionsmedium Wasser oder ein Gemisch von Wasser mit organischem Lösungsmittel, welches mindestens 10 Gew.-% Wasser umfasst, ist.

WO 02/080979 A3

WO 02/080979 A3



CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

OAPI-Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Veröffentlicht:**

— mit internationalem Recherchenbericht

**(88) Veröffentlichungsdatum des internationalen**

**Recherchenberichts:**

12. September 2003

**(84) Bestimmungsstaaten (regional):** ARIPO-Patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IT, IJ, MC, NL, PT, SE, TR),

*Zur Erklärung der Zweibuchstaben-Codes und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.*

CONJUGATE OF HYDROXYALKYL STARCH  
AND AN ACTIVE AGENT

The present invention relates to compounds comprising a conjugate of hydroxyalkyl starch (HAS) and an active ingredient, wherein the hydroxyalkyl starch is coupled to the active ingredient either directly or via a linker. The invention further relates to processes for the preparation of a covalent HAS-active ingredient conjugate in which HAS and an active ingredient are reacted with each other in a reaction medium, wherein the reaction medium is water or a mixture of water with an organic solvent, having at least 10 weight-% water. The invention further relates to the medical use of the conjugates.

TECHNICAL BACKGROUND

The clinical use of many active ingredients of pharmaceuticals is adversely affected by a number of problems (cf. Delgado et al., Critical Reviews in Therapeutic Drug Carrier Systems, Vol. 9 (3, 4), (1992) pp. 249-304). Parenterally administered native proteins are subject for example to excretion by the reticuloendothelial

system, the liver, the kidney and the spleen. Excretion depends on the charge of carbohydrate chains, the presence of cellular receptors for the protein and molecule shape and size. The excretion limit of the glomerular filtration of the kidney is for example approx. 67 kD.

As a result of proteolytic degradation, a rapid loss of biological activity can also be observed.

Proteins expressed by bacteria as well as other recombinant proteins can have an increased immunogenicity and provoke life-threatening hypersensitivity reactions. Corresponding reactions naturally prevent the medical use of these products.

For this reason, research has been carried out systematically in the state of the art already since the end of the 70s on the improvement of the properties of exogenic proteins by chemical modification, in particular polymerization or coupling to macromolecular polymers. Many projects concentrated on the preparation of conjugates from proteins or other active ingredients on the one hand and polyethylene glycol (PEG) on the other (cf. US 4,179,337). The advantages expected from respective coupling reactions comprise improved in vivo half-life of the proteins, reduced toxicity, improved stability and improved solubility of the active ingredients (Abuchowski and Davis, Enzymes as drugs, Holcenberg and Rubberts, Publisher, pp. 367-383, John Wiley & Sons N.Y. (1981)).

The process of coupling the active ingredients proved to be problematic however, as the active group of the protein was inactivated by coupling to PEG or the reactions did not provide the reaction product in a suitable yield. To achieve a specific coupling which does not adversely affect the activity of the active ingredient, active groups were introduced into PEG or the

active ingredient or the compounds were coupled with a linker. For this purpose, PEG is normally provided with an active group which is subsequently covalently bound to a group of a protein capable of being coupled.

Thus for example, the loss of the binding activity of antibodies and their fragments after their coupling to PEG was described (Kitamura et al., Cancer Res., Vol. 51 (1991), pp. 4310-4315; and Pedley, et al., Br. J. Cancer, Vol. 79 (1994), pp. 1126-1130). To solve this problem, Chapman et al. (Nature Biotech., Vol. 17 (1999), pp. 780-783) suggest binding PEG to certain binding regions of the antibody.

The loss of activity of the coupling partner is also described in WO 95/13090. As a solution, it is suggested to activate PEG with a reactive group and to bind PEG to  $\alpha$ -interferon in the presence of a surfactant via this reactive group. Cited as preferred reactive group is N-succinimide carbonate, which is said to form a urethane bond with the  $\epsilon$ -amino group of lysine under the conditions named.

WO 96/41813 also discloses processes for the preparation of a polymer-polypeptide conjugate in which the polymer (in particular PEG) is derivatised at a specific region and then bound to a polypeptide. An amino-oxi-acetyl group is preferably introduced into PEG and this compound is then bound to a polypeptide, in particular to IL-8, hG-CSF and IL-1.

In the literature, there are thus numerous examples of corresponding conjugates; cf. PEG-insulin conjugates in US 4,179,337, PEG-bovine-haemoglobin conjugates in US 4,412,989, PEG-ribonuclease conjugates and PEG superoxide dismutase conjugates in Veronese et al. Applied Biochem. Biotech., Vol. 11, 141-152 (1995), PEG-IL-2 conjugates or PEG-IFN- $\beta$  conjugates in US 4,766,106, PEG-polymyxin conjugates in WO 90/15628 and PEG-IL-2

conjugates in WO 90/07939. Some conjugates are now in clinical application. For example, the properties of the enzyme asparaginase were improved by conjugate formation with PEG, and a PEG-asparaginase conjugate is commercially available under the trademark Oncaspar® for cancer therapy. Recently, a PEG-coupled G-CSF was approved by the US Food and Drug Administration (Pegfilgastim). A large number of further pegylated products are in different phases of clinical development, for example PEG-CDP870, PEG-Dronabinol, etc. (cf. PEG-pipeline at [www.enzon.com](http://www.enzon.com) or [www.inhale.com](http://www.inhale.com)).

Not only proteins, but other compounds were also coupled to PEG and other polymers according to this scheme. WO 97/33552 and WO 97/38727 disclose for example the coupling of paclitaxel to PEG and the use of the conjugate for the treatment of tumors. The use of a PEG-camptothecin conjugate for the treatment of tumors is being studied by Enzon in phase I clinical trials.

Antibiotics have also been coupled to PEG. Dowling and Russell, for example, describe the pharmacokinetics of an oxytetracyclin-PEG conjugate (J. Vet. Pharmacol. Ther., vol. 23 (2000), 107 - 110). In the state of the art, antibiotics have also been derivatized using other methods in order to obtain new functions. For example, a depot penicillin was produced, which is a procain-penicillin derivative, i.e. a salt of the penicillin with the procain base. This derivative has an extended activity and it is used, for example, in the therapy of Syphilis.

Coupling reactions with more than two compounds have also been demonstrated. For example, WO 93/23062 discloses the preparation of a coupling product from an antibody directed against a B cell lymphoma, activated PEG and a toxin.

PEG-active ingredient conjugates however do not have a natural



structure for which in vivo decomposition pathways have been described. Amongst others for this reason, in addition to the PEG conjugates, other conjugates and protein polymers have been produced for solving the above-named problems. Thus, there are a number of processes for cross-linking different proteins and binding of proteins to macromolecules (cf. summary in Wong, S.S., "Chemistry of protein conjugation and cross linking", CRCs, Inc. (1993)).

Hydroxyethyl starch (HES) is a derivative of a naturally occurring amylopectin and is broken down in the body by  $\alpha$ -amylase. The preparation of HES-protein conjugates has already been described in the state of the art (cf. HES-haemoglobin conjugates in DE 26 16 086 or DE 26 46 854).

Haemoglobin is a protein which could be of great clinical importance as a blood-replacement and oxygen-carrier agent (so-called Haemoglobin-Based-Oxygen Carrier, HBOC). However, although the demand for such a product was recognized early on (cf. Rabiner, J. Exp. Med. 126, (1967) 1127), none of the known HBOC products has as yet achieved the status of an approved drug.

The natural haemoglobin consists of two  $\alpha$  and  $\beta$  peptide chains which each bind a haeme as a prosthetic group. Isolated haemoglobin molecules are however very unstable and rapidly break down into the more stable  $\alpha, \beta$  dimers (MW 32 kDa). The biological half-life of isolated haemoglobin in the blood circulation is approx. 1 hour, as the dimers are rapidly eliminated via the kidneys. In this process, the dimers produce nephrotoxic side effects (cf. Bunn & Jandl, J. Exp. Med. 129, (1967) 925-934). Development work on derivatized haemoglobin molecules was therefore primarily directed towards the intramolecular cross-linking of haemoglobin, the intermolecular cross-linking to form polymeric HBOC forms and/or the coupling to polymers.

The known haemoglobin conjugates are described for example in Xue and Wong (Meth. in Enzymol., 231 (1994), pp. 308-322) and in DE 26 16 086 and DE 26 46 854. The latter discloses processes by means of which haemoglobin is bound to HES by firstly reacting HES with sodium periodate. Dialdehydes form, to which haemoglobin is bound. On the other hand, DE 26 16 086 describes the coupling of haemoglobin to HES according to a process in which firstly a cross-linking agent (e.g. Bromcyan) is bound to HES and haemoglobin is then bound to the intermediate product.

HES is a substituted derivative of the carbohydrate polymer amylopectin which occurs in maize starch in a concentration of up to 95%. HES has advantageous rheological properties and currently used in the clinic as a volume-replacement agent and for haemodilution therapy (Sommermeyer et al., Krankenhauspharmazie, Vol. 8(8), (1987), pp. 271-278; and Weidler et al., Arzneim.-Forschung/Drug Res., 41, (1991) 494-498).

Amylopectin consists of glucose units, wherein the main chains have  $\alpha$ -1,4-glycosidic bonds, but  $\alpha$ -1,6-glycosidic bonds are present at the branching sites. The physical-chemical properties of this molecule are determined essentially by the type of glycosidic bonds. Because of the branched  $\alpha$ -1,4-glycosidic bond, helical structures form with approx. 6 glucose monomers per turn.

The physico-chemical and the biochemical properties of the polymer can be modified by substitution. The introduction of a hydroxyethyl group can be achieved by alkaline hydroxyethylation. The different reactivity of the relevant hydroxyl group in the unsubstituted glucose monomer vis-à-vis the hydroxyethylation can be exploited through the reaction conditions, a limited influence on the substitution pattern is thus possible.

HES is therefore essentially characterized via a molecular weight distribution and a degree of substitution. The degree of substitution can be described as DS "degree of substitution" which refers to the proportion of the substituted glucose monomers of all glucose units, or as MS ("molar substitution"), which gives the number of hydroxyethyl groups per glucose unit.

HES solutions are present as polydisperse compositions in which the individual molecules differ from each other with regard to the degree of polymerization, the number and arrangement of the branching sites, as well as their substitution pattern. HES is thus a mixture of compounds with different molecular weights. Accordingly, a specific HES solution is determined by an average molecular weight using statistical variables.  $M_n$  is calculated as a simple arithmetic average in relation to the number of molecules (numerical average), whilst  $M_w$ , the weight average, represents the mass-related measurement variable.

A selective chemical binding of proteins to HES was however hitherto prevented by the fact that the HES is not activated selectively. Thus the protein-HES conjugates known in the state of the art result from a non-selective coupling of Bromcyan-activated HES to haemoglobin (cf. DE 26 16 086). Corresponding processes can lead to polydisperse products with non-uniform properties and potentially toxic side effects.

A process was first disclosed by Hashimoto (Hashimoto et al., *Kunststoffe, Kautschuk, Fasern*, Vol. 9, (1992) pp. 1271-1279) wherein the reducing aldehyde end group of a saccharide is selectively oxidized and a reactive ester (lactone) is obtained.

On the basis of this process, WO 98/01158 discloses that haemoglobin-hydroxyethyl starch conjugates can be obtained in which haemoglobin and HES are selectively linked to each other via

amide bonds between free amino groups of the haemoglobin and the reducing end group of the HES present in oxidized form. Both the processes described in Hashimoto et al. and the processes according to WO 98/01158 are however based on a reaction between saccharide (HES) and protein (haemoglobin) in organic solvent. Dimethyl sulfoxide (DMSO) was in fact used in the publication.

One of ordinary skill in the art is aware of the fact that many proteins are subject of a change in structure in organic solvents which is not reversed in aqueous solution. Regularly, a loss of activity occurs with the change in structure. In every case, a costly removal of the organic solvent is necessary, as even residual proportions of organic solvents may not be acceptable for the intended medical use. Even the potential danger of impurities and changes in structure of the proteins is to be excluded with regard to the intended use.

The object of the present invention is thus to provide improved hydroxyalkyl starch-active ingredient conjugates and processes for their preparation which lead to biologically active conjugates which can be used in everyday clinical practice. A further object of the present invention is to provide a process for the preparation of hydroxyalkyl starch-active ingredient conjugates wherein by-products are not produced in significant quantities, as these by-products also adversely affect the subsequent purification of the product to a significant extent.

This object was now surprisingly solved by compounds comprising a conjugate of hydroxyalkyl starch and an active ingredient, wherein the hydroxyalkyl starch is covalently bound to the active ingredient either directly or via a linker. Corresponding HAS-active ingredient conjugates are for example obtainable by processes, wherein HAS and an active ingredient are coupled in a reaction medium, wherein the reaction medium is water or a mixture

of water with an organic solvent, which comprises at least 10 weight-% water.

The invention further relates to processes for the preparation of a covalent HAS-active ingredient conjugate, wherein HAS and at least one active ingredient are coupled in an aqueous reaction medium and is characterized in that the reaction medium is water or a mixture of water with an organic solvent, which comprises at least 10 weight-% water.

HAS is preferably oxidized before binding to the active ingredient, a specific oxidation of the reducing end groups being particularly preferred. Alternatively, the coupling can take place via the formation of a Schiff's base between HAS and an amine group-carrying active ingredient as intermediate product. This intermediate product is then reduced, resulting in the formation of a methylene amine group.

#### BRIEF DESCRIPTION OF THE FIGURES:

- Fig. 1     GPC chromatogram of the coupling reaction between ox-HES 130 kD and HSA according to process A.III;
- Fig. 2     GPC chromatogram of the coupling reaction between ox-HES 130 kD and HSA according to process A.IV;
- Fig. 3     GPC chromatogram of the coupling reaction between ox-HES 130 kD and HSA according to process A.V. and with a reaction time of 2 hours;
- Fig. 4     GPC chromatogram of the coupling reaction between ox-HES 130 kD and HSA, process A.V., reaction time overnight;

- Fig. 5 GPC chromatogram of the coupling reaction between ox-HES 10 kD and HSA according to process A.V, after 2 hours (Fig. 5a) and overnight (Fig. 5b);
- Fig. 6 GPC chromatogram of the coupling reaction between ox-HES 130 kD and HSA according to process A.VII, after 24 hours reaction time;
- Fig. 7 GPC chromatogram of the coupling reaction between ox-HES 130 kD and HSA according to process B.V;
- Fig. 8 SDS-PAGE and Western Blot of different coupling reactions between HES and HSA;
- Fig. 9 SDS-PAGE and Western Blot of different coupling reactions between HES and HSA;
- Fig. 10 reaction scheme for the preparation of an HES-DNA conjugate;
- Fig. 11 image of a gel showing the HES-DNA conjugate prior to and after digestion with a restriction enzyme.

The present invention provides for the first time compounds comprising a conjugate of hydroxyalkyl starch and an active ingredient, wherein the hydroxyalkyl starch is covalently bound to the active ingredient either directly or via a linker. The present invention further provides HAS-active ingredient conjugates which can be prepared by processes, wherein HAS and at least one active ingredient are reacted with each other in an aqueous reaction medium. The processes are further characterized in that the reaction medium is water or a mixture of water with an organic solvent, which comprises at least 10 weight-% water.

Within the context of the present invention, a chemical compound is referred to as an active ingredient if the compound is suitable to be an active component of any composition for therapeutic or diagnostic purposes. Preferably, the active ingredient is an active component of a drug, i.e. the compound in a drug formulation which achieves a physiological effect after administration to a subject.

An overview of the approved drugs and their active ingredients is given in the pharmacopeia. All the active ingredients named in the pharmacopeia can be used for the preparation of the HAS-active ingredient conjugates by the process according to the invention. However, according to the present invention, the term active ingredient also comprises all compounds which, although known to be suitable for diagnostic or therapeutic use, were however not able to be used up to now for this purpose, because of the problems described above. The active ingredient is preferably a vitamin, vaccine, toxin, antibiotic (or antiinfective), antiarrhythmic, appetite suppressant, anesthetic, analgesic, antirheumatic, antiallergic, antiasthmatic, antidepressant, antidiabetic, antihistamine, antihypertonic or an antineoplastic agent. Structurally, it can be for example a hormone, steroid, lipid, protein, oligo- or polypeptide, a nucleic acid, in particular a D- or L-nucleic acid, such as a D-DNA, L-DNA, D-RNA or L-RNA. The use of proteins, peptides, D- or L-nucleic acids as HAS coupling partners is particularly preferred.

The compounds prepared according to the present invention retain the activity of the active ingredient and the advantageous properties of the HAS. As further advantages, the conjugates prepared according to the process according to the invention have an improved in vivo half-life of the active ingredients, reduced toxicity, improved stability and/or improved solubility of the

active ingredients.

After administration, the HAS chain is shortened by the  $\alpha$ -amylase in the plasma. Thus, the activity of the coupling product can be determined as activity of the native coupling product, i.e. directly after the coupling, or as activity of the metabolized coupling product, i.e. after in vivo metabolizing of the coupling product. In vivo metabolizing can be simulated by an in vitro degradation.

The activity of the active ingredient be determined by methods which are known for this compound in the state of the art. For example, the activity of an antineoplastic agent is determined as inhibitory concentration (IC), and the activity of an antiinfective agent is determined as minimal inhibitory concentration (MIC). Preferably, the determination is performed in vitro with appropriate target cells (cf. Chow et al., Haematologica, Volume 86 (2001), pages 485-493). The in vitro effects can further be confirmed by a relevant animal model (cf. for example the mouse model of the renal cell carcinoma described in Changnon et al., cf. BJU Int., Volume 88 (2001), page 418-424).

Compared to the non-coupled substance, the native coupling product can have an increased or reduced activity. Preferably, however, the activity is not reduced more than 5-fold, more preferably not more than 3- or 2-fold. The metabolized product preferably has an activity comparable to that of the non-coupled substance, i.e. prior to the coupling, the metabolized conjugate has at least 50 %, preferably at least 75 % of the activity of the active ingredient, wherein a retention of at least 95 % of the activity is particularly preferred.



In the context of the present invention, the term "hydroxyalkyl starch" is used to refer to starch derivatives which are substituted with a hydroxyalkyl group having 1 to 3 carbon atoms. Thus, the group designated as "hydroxyalkyl starch" comprises hydroxymethyl starch, hydroxyethyl starch and hydroxypropyl starch. The use of hydroxyethyl starch (HES) as a coupling partner is particularly preferred for all embodiments of the invention.

According to the invention, it is preferred that the hydroxyethyl starch has an average molecular weight (weight average) of 1-300 kDa, wherein an average molecular weight of 5 to 200 kDa is particularly preferred. Furthermore, hydroxyethyl starch may have a molar degree of substitution of 0.1 to 0.8 and a ratio of  $C_2:C_6$ -substitution in the range of 2-20, in each case relative to the hydroxyethyl groups.

For coupling the active ingredient to the HAS, it may be necessary in a first step to introduce an active group into the active ingredient and/or the HAS. Corresponding active groups can for example be thiol groups or amino groups (cf. Examples).

Further, the active ingredient and the HAS can be coupled to each other by use of a linker. Any crosslinking agent can be used as a linker. Numerous crosslinking agents such as SMCC (succinimidyl-4-(N-maleimido-methyl)cyclohexane-1-carboxylate; cf. Example 7) are commercially available and well-known to the person skilled in the art (cf. alphabetic list of the "cross-linking reagents" in the product catalogue of the company Perbio and [www.piercenet.com](http://www.piercenet.com) ).

According to a further embodiment of the present invention, water-soluble antibiotic derivatives which contain an amino sugar, in particular HAS-daunorubicin and HAS-doxorubicin

conjugates, and processes for their preparation, as far as they are disclosed in DE 101 29 369, are not comprised by the scope of the present invention.

According to a preferred embodiment, the present invention relates to compounds comprising a conjugate of HAS and an antineoplastic active ingredient and their use for the treatment of tumors.

Among others, tumor cells differ from normal somatic cells in that tumor cells are no longer subject to a physiological growth control and therefore have an increased rate of cell division. The therapeutic use of antineoplastic active ingredients in tumor therapy is based on this difference, since the toxic activity of the antineoplastic active ingredients is primarily directed against proliferating cells. As a consequence, compounds are designated as antineoplastic active ingredients or cytostatics if they exhibit a toxic activity against proliferating cells (basics of oncology and current therapeutic approaches are for example summarized in: Internistic Oncology, Schmoll et al. (eds.), Springer, 1996).

With respect to their chemistry, antineoplastic active ingredients represent a very heterogeneous group. In addition to the inhibition of proliferation, the induction of apoptosis, programmed cell death, gains importance in the discussions over the last years. A classification of the antineoplastic active ingredients can for example be performed based on the relevant target molecules (Schmoll et al., see above):

1. Compounds which inhibit DNA biosynthesis, for example as antimetabolites, such as MTX, 5-FU, Ara-C or hydroxy urea.
2. Compounds, which act on the DNA, for example by strand break induction, intercalation, modification of interstrand cross-

linking, topoisomerase toxins, such as alkylating agents, platinum complexes, anthracyclins, bleomycin, actinomycin-D or epipodophyllo toxins.

3. Compounds which act on the RNA, for example by blocking mRNA-synthesis by intercalation or incorporation into the RNA, including anthracyclins, bleomycin, actinomycin-D or antimetabolites.
4. Compounds, which act on proteins, for example on the level of receptor binding (e.g. hormones or antagonists), by inhibition of tubulin polymerization (e.g. by vinca alkaloids), by protein cross-linking (for example by alkylating agents) or phosphorylation (e.g. by inhibitors of protein kinase C).

Due to the antineoplastic activity, all active ingredients have considerable side effects, which primarily occur as inhibition of fast proliferating tissues. For this reason, in particular erythro-, leuko- and trombopoiesis are inhibited and the growth of mucous membrane epithelia is adversely affected. As a consequence, gastrointestinal disorders or non-reversible impairments of spermatogenesis or anovulation, respectively, can occur. The skin and the skin accessory organs are also usually affected. For example, many patients suffer from a reversible loss of hair.

In severe cases, the side effects can lead to an acute loss of the kidney function and toxic-related organ damages to heart, lung, liver and nervous system. Finally, as a consequence of the immunosuppressive effect, an increased number of infections has to be expected.

The preparation and investigation of conjugates which contain an antineoplastic agent were therefore focused on the improvement of the tolerance of the active ingredient. For this purpose,

different antineoplastic active ingredients have been coupled to macromolecules such as dextran (cf. Kojima et al., J. Pharm. Pharmacol., Vol. 32 (1980), p. 30-34; Nakane et al., J. Pharm. Pharmacol., vol. 40 (1988), p. 1-6, Nomura et al., J. Controlled Release, Vol. 52 (1998), p. 239-252; Sato et al., J. Pharm. Sci., Vol. 78 (1989), p. 11-16). In several cases, an improved anti-tumor effect of the conjugates was demonstrated.

As an alternative, active ingredients such as mitomycin C were also coupled to N-succinylchitosan (Song et al., J. Controlled Release, Vol. 42 (1996), p. 93-100), carboxymethylchitin (Song et al., Arch. Pract. Pharm. Vol. 53 (1993), p. 141-147) and oligopeptides (Soyez et al., J. Controlled Release, Vol. 47 (1997), p. 71-80). When compared to the individual antineoplastic active ingredient, again, an improved anti-tumor activity of the conjugates was observed in the majority of analyses.

According to the invention it was now surprisingly found, that HAS-active ingredient conjugates which comprise an antineoplastic active ingredient have an improved toxic effect against tumor cells and/or a reduced toxicity for other cells. Therefore, the conjugates allow for a broader therapeutic range.

The plasma half-life of the conjugates is significantly increased. This allows to overcome the repair mechanisms in tumor cells by longer exposition. Simultaneously, the present invention enables slower flooding, in particular in healthy tissue, whereby a reduced peak concentration and an improved tolerance for the patient is achieved.

For the preparation of the conjugates according to the invention, any antineoplastic active ingredient can be used. The antineoplastic active ingredient can, for example, be selected from the group consisting of alkylating agents, antimetabolites,

antibiotics or natural substances.

According to a preferred embodiment, the antineoplastic active ingredient is mitomycin C, cyclophosphamid, bleocin, chlorambucil, cisplatin, Ara-C, fludarabine, doxorubicin, etoposide, 5-FU, MTX, vinblastine, vincristine, vindesine, hydroxy urea, 6-MP or CCNU.

The use of mitomycin C as active ingredient is particularly preferred. Mitomycin C belongs to the group of antibiotics and contains an aziridine group and a quinone group and a mitosane ring. The active ingredient is used for the treatment of renal cell carcinoma and bladder tumors as well as other urologic diseases. The compound gains its activity only upon metabolization in hypoxic cells (this means preferably in tumor cells) by intracellular enzymatic or spontaneous chemical reduction of the quinone and loss of the methoxy group. Preferably, HAS can be coupled to this methoxy group via a linker. After intracellularly cleaving off the substituent, the same active ingredient is present inside the cell which causes an alkylating cross-linking of the DNA thereby exhibiting its toxic effect. As an alternative, HAS may also be coupled to one of the two  $\text{NH}_2$ -groups. Mitomycin C shows a typical tissue specificity. According to the invention, it is preferred that this specificity - in particular for excretory organs - is increased by HAS-coupling.

According to the invention, the antineoplastic active ingredient can be coupled to HAS by use of any method. However, a specific coupling to the reducing end groups of HAS is preferred, since this procedure generates a defined conjugate.

According to one embodiment of the invention, hydroxyethyl starch may be coupled to the methoxy group of mitomycin C. Coupling to

the methoxy group of mitomycin C can take place via a linker.

According to a further embodiment, the present invention relates to processes for the preparation of a compound comprising a conjugate of HAS and an antineoplastic active ingredient. The process comprises steps, in which HAS is covalently coupled to an antineoplastic active ingredient, either directly or via a linker, and the conjugate is isolated.

Further, the invention relates to pharmaceutical compositions which comprise a compound comprising a conjugate of HAS and an antineoplastic active ingredient. The pharmaceutical composition can furthermore comprise a pharmaceutically compatible carrier and/or a cytokine. Preferably, the cytokine is IL-2,  $\alpha$ -interferon,  $\gamma$ -interferon.

The pharmaceutical composition can be in any application form which is known in the state of the art. For example, the composition can be formulated for oral or parenteral administration. The formulation of the composition is performed according to processes known in the state of the art. In addition to the active ingredient, the composition generally comprises a pharmaceutically compatible carrier and one or more auxiliaries and optionally preservatives, solubility promoters, etc.

Finally, the present invention relates to the use of a compound comprising a conjugate of HAS and an antineoplastic active ingredient for the preparation of a medicament for the treatment of tumors and/or their metastases, in particular for the treatment of urologic tumors and/or metastases of urologic tumors, for the treatment of metastases of the renal cell carcinoma, or for the treatment of diseases of the lymphatic system, such as CLL, Hodgkin-lymphoma, NHL, multiple myeloma,

Waldenström's syndrome. According to this embodiment of the invention, the medicament can further comprise a cytokine, such as IL-2,  $\alpha$ -interferon,  $\gamma$ -interferon.

The use of the compounds according to the invention for the preparation of a medicament for the treatment of urologic tumors and/or metastases of urologic tumors, such as for the treatment of metastases of the renal cell carcinoma is particularly preferred. Presently, a curative therapy of the renal cell carcinoma can neither be achieved with a combination chemotherapy nor with mitomycin C alone. This might be due to the unfavourable pharmacokinetics of the compound, since the portion of renal elimination only amounts to approximately 18 %. Since HAS is almost completely eliminated via the kidney, the conjugate exhibits a higher percentage of renal elimination compared to the non-conjugated substance. This embodiment of the present invention utilizes the intracellular intermediate storage of HAS. In particular, highly substituted HAS species (HAS 200/0.62) show an increased intracellular storage, in the extreme case even an overload. This phenomenon has also been observed in the area of the proximal tubule (Peron et al., Clinical Nephrology, Vol. 55 (2001), p. 408-411).

According to this embodiment, the present invention provides an accumulation of an antineoplastic active ingredient in certain target cells or tissues. Therefore, the improved pharmacokinetics of the conjugates make it possible to achieve a considerably higher concentration in the cells of the target organ while using low systemic concentrations. This medical use is preferably employed on the hypernephroid carcinoma and the chromophylic renal carcinoma which constitute approximately 90 % of all histological types.

According to an alternative embodiment, the invention relates to the use of the compounds according to the invention for the preparation of a medicament for the treatment of diseases of the lymphatic system, such as CLL, Hodgkin lymphoma, NHL, multiple myeloma, Waldenström's syndrome. By coupling of HAS to an antineoplastic active ingredient according to the invention, the intracellular uptake of the active ingredients is decelerated dependent on the chain length and the degree of substitution. Furthermore, radioactive kinetic studies have shown that HAS is stored in certain organs, among others in lymphatic organs, for a longer time than in the whole body (cf. Bepperling et. al., Crit. Care, Vol. 3, Suppl. 1 (1999), p. 153). Thus, accumulation of the conjugate in the target cells occurs which results in improved pharmacokinetics with a lower systemic toxicity.

The treatment of diseases of the lymphatic system using fludarabin as an active ingredient is preferred. Fludarabin is a halogenated adenine analogue which is resistant to deamination.

The invention further relates to the use of the compounds according to the invention for the preparation of a medicament for the treatment of cutaneous/local primary malignant neoplasms or their metastases. For this, two effects can be utilized, the directed increased uptake by the recited tissues and the decelerated transport of the HAS conjugates out of the tissue. Both effects lead to an accumulation of the conjugate in the target cells.

The invention further relates to the use of the compounds according to the invention for the preparation of a medicament for the treatment of diseases of the hematologic system or oncologic diseases, such as non-small cell lung cancer and small cell lung cancer, breast cancer, esophagus squamous cell carcinoma, renal cell carcinoma, testicular carcinoma, malignant



melanoma, ALL or CML. In particular, when using the conjugates for the treatment of the renal cell carcinoma, advantages arise due to the strong accumulation of the compound in the affected tissue by the increased hydrophilicity of the conjugate and the stronger renal elimination resulting thereof. For this embodiment of the invention, the use of vindesine as active ingredient is particularly preferred.

The invention further relates to the use of the compound according to the invention for the preparation of a medicament, wherein the compound is used as a combination therapy with one or more further antineoplastic active ingredients or cytokines. The combination therapy can be performed by administration of an agent containing all active ingredients, or by administration of two or more different compositions, each of which containing one active ingredient.

The present invention further provides processes for the preparation of a medicament comprising a cytokine and a compound according to the invention which is suitable for new combination therapies. Corresponding agents are in particular suitable for the treatment of the advanced renal cell carcinoma.

According to another particularly preferred embodiment of the invention, conjugates of HAS and an antiarrhythmic active ingredient as well as their use for the treatment of arrhythmia are provided.

Deviations from the temporary sequence and regularity of the heart beat (arrhythmia) from the normal heart rate are referred to as arrhythmia. In the majority of cases, these deviations are caused by cardiac excitation or conduction disorders. Substances which are suitable for the treatment of arrhythmia, in particular ventricular arrhythmia, are referred to as antiarrhythmic active

ingredients or antiarrhythmics.

Dependent on the effect of the antiarrhythmic active ingredients it is distinguished between sodium channel blockers (quinidine, procainamide, disopyramide, etc.) beta-receptor blockers (atenolol, propranolol, etc.), selective repolarisation prolonging active ingredients (amiodarone, sotalol, etc.), calcium antagonists (verapamil, gallopamil, etc.) and local anesthetics.

However, the antiarrhythmic active ingredients customary in the state of the art partially exhibit a short duration of action. For example, adenosine is an antiarrhythmic active ingredient with a very short half-life. The duration of action of this substance is only several minutes. In many cases, prolongation of half-life and duration of action is necessary.

Additionally, several antiarrhythmic active ingredients have proarrhythmogenic side effects and partially even an increase in mortality.

The present invention provides, among others, improved antiarrhythmic active ingredients which, for example, have a prolonged duration of action. According to the invention, it was surprisingly found that the HAS-antiarrhythmic conjugates have a significantly longer in vivo plasma half-life and that the activity of the active ingredients is not adversely affected to a significant extent by coupling to HAS.

According to the present invention, any antiarrhythmic active ingredient can be used for the preparation of the conjugates. The active ingredient can be selected from the group consisting of sodium channel blockers, beta-receptor blockers, selective repolarization prolonging active ingredients, calcium antagonists and local anesthetics. Preferably, the active ingredient is

---

adenosine, quinidine, procainamide, disopyramide, lidocaine, phenytoin, mexiletine, ajmaline, Parjmalium, propafenone, atenolol, propranolol, amiodarone, sotalol, verapamil, gallopamil or diltiazem, wherein the use of adenosine is particularly preferred.

According to an embodiment of the present invention, coupling between the antiarrhythmic active ingredient and the HAS takes place via the reducing end groups of the HAS.

When adenosine is used, this active ingredient can for example be bound to the HAS via the amino group, wherein a coupling between the amino group of the adenosine and the reducing end group of the HAS is particularly preferred. A coupling variant, wherein native adenosine is present after metabolism (separating off the HAS) is preferred.

As an alternative, the active ingredient can be coupled to the HAS via a so-called linker.

The present invention further relates to pharmaceutical compositions comprising one of the compounds according to the invention. Generally, the pharmaceutical composition further comprises a pharmaceutically compatible carrier, and it can be formulated, for example, for intravenous application.

Finally the invention relates to the use of the compounds according to the invention for the preparation of a medicament for the treatment of arrhythmia, in particular for the treatment of ventricular arrhythmia.

According to an alternative embodiment, the invention relates to the use of a compound according to the invention for the preparation of a medicament for the induction of apoptosis, for

example in tumor tissues or in inflammatory tissues.

The present invention relates to compounds comprising a conjugate of HAS and an antiinfective active ingredient or an antibiotic, respectively, as well as their use for the treatment of infectious diseases.

The penetration of microorganisms (viruses, bacteria, fungi, protozoa) into a macroorganism (plant, animal, human) and the propagation in this macroorganism is called infection. Formation and course of an infectious disease substantially depend on pathogenicity of the microorganism and immunity of the macroorganism.

For decades, antiinfective active ingredients has been used as chemotherapeutics in order to fight infectious diseases.

A. Flemings identified Penicillin already in 1928 by the active ingredient's characteristic to form staphylococci-free areas on culture plates. Penicillin was the first antibiotic which was obtained in industrial scale and it gained big importance in clinical practice.

Today, active ingredients from a group of  $\beta$ -lactam antibiotics which are produced from a fungus of the species *Penicillium* (for example *P. chrysogenum* und *P. notatum*) are designated as penicillins. The bacteriocidal effect is based on blocking the synthesis of the bacterial cell wall. The penicillin inactivates the bacterial enzyme transpeptidase, thereby preventing cross-linking of the polysaccharide chains of the cell wall murein.

Since the discovery, numerous active ingredients were isolated and synthesized which inhibit the growth of microorganisms or

kill microorganisms. Most antibiotics originate from *Streptomyces* species (approximately 65 %) which were isolated from soil. It is assumed that these substances are used by the microorganism to suppress competitors in the soil.

The number of isolated antibiotics is estimated to be approximately 8000, approximately 100 thereof can be used in the field of medicine. A classification of the active ingredients into different substance classes was performed according to different aspects, for example chemical structure or mode of action.

Meanwhile, antibiotics are approved not only for fighting infectious diseases, but also as immuno depressants, cytostatics in anti-tumor therapy, plant protectives, for the preservation of foods, as fattening auxiliary agent in the feeding of animals, etc.

In recent years, numerous strains of microorganisms occurred which are resistant to antibiotics. In addition to single-resistant strains, multi-resistant strains were frequently found which complicates fighting of certain diseases.

When studying the activity of different antibiotics against certain pathogens, it was found that several of the active ingredients, for example amoxycillin or ampicillin, almost exclusively act extracellularly (Scaglione et al., *Chemotherapie*, Vol. 39 (1993), 416-423; Balland et al., *J. Antimicrob. Chemother.* Vol. 37 (1996), 105-115). Therefore, these active ingredients cannot be used against microorganisms which primarily are present inside the cell. Ampicillin-nanoparticles have been produced in order to improve the intracellular activity (cf. Balland et. al., see above).

For infections such as tuberculosis or other infections caused by mycobacteri, broadening of the spectrum of treatment possibilities would be desirable due to the combination therapy which is always required. In view of other intracellular infections such as chlamydia infection, for which the potential importance for the pathogenesis of arteriosclerosis was only recently discovered (Stille und Dittmann, Herz, Vol. 23 (1998), p. 185-192), intracellular antibiotics with a depot effect could represent an important progress in therapy and prophylaxis.

According to the invention, it was now surprisingly found that coupling of antiinfective active ingredients to HAS results in improved pharmacokinetic characteristics of the active ingredients, in particular in a prolonged in vivo half-life, an improved intracellular uptake and/or effect of the active ingredient.

According to the invention, any antiinfective ingredient or antibiotic, respectively, can be used. Preferably, an active ingredient is selected from the group consisting of amino penicillins, cephalosporines, amino cephalosporines, beta-lactam-antibiotics, carbapenems, amino glycosides, tetracyclines, macrolide antibiotics, gyrase inhibitors, glycopeptide antibiotics, lincomycins, streptogramins, everninomicins, oxazolidinones, nitroimidazoles, sulfonamides, co-trimoxazol, local antibiotics, virustatics, antimycotics, tuberculostatics.

It may for example be ampicillin, amoxicillin, cefotaxim, ceftazidim, vancomycin, clindamycin, metronidazol, isoniazid, rifampicin, rifabutin, rifapentin, ethambutol, pyracinamide, streptomycin, prothionamide, or dapsone, wherein the use of an amino penicillin, such as ampicillin, amoxycillin, macrolide or of streptomycin is particularly preferred.

---

According to one embodiment of the present invention, an amino penicillin is used as an active ingredient which is directly and covalently coupled to the hydroxyethyl starch via the amino group of the amino penicillin.

According to another embodiment, an amino cephalosporin is used instead of the amino penicillin, thereby achieving a reduced allergenicity. As further embodiments, macrolide-HAS couplings may be used, wherein erythromycin or a derivative thereof is used, in particular erythromycylamin. As an alternative, streptomycin can be used as active ingredient.

According to a particularly preferred embodiment of the present invention, the coupling between the antiinfective active agent and the hydroxyethyl starch may take place via the reducing end groups of the hydroxyethyl starch.

In accordance with a further embodiment of the present invention, the antiinfective active agent is coupled to the hydroxyethyl starch via a linker.

The present invention further comprises pharmaceutical compositions, which comprise a compound according to the invention. Usually, the pharmaceutical compositions further comprise a pharmaceutically compatible carrier.

Finally, the present invention relates to the use of one of the compounds according to the invention for the preparation of a medicament for the treatment of an infectious disease. The pharmaceutical composition may in particular be suitable for the treatment of infectious diseases which, amongst others, are caused by intracellular pathogens. These may originate from the complete spectrum of pathogenics and facultative pathogenics, for example bacterial, viral or parasitic pathogens, mycoplasmas,

mycobacteria, chlamydia, rickettsia, etc.

In a further aspect of the present invention, HAS-nucleic acid conjugates are provided. Presently, nucleic acid libraries are screened in large scale for nucleic acids which have a desired activity. For example, a respective activity can be the ability of a nucleic acid to bind to certain other nucleic acids, receptors or viral proteins. This binding may be stimulated or inhibited by a biological signal. For this purpose, in addition to naturally occurring D-DNA and D-RNA molecules, also L-DNA and L-RNA molecules are used which differ from the naturally occurring molecules in that they contain L-ribose or L-deoxyribose instead of the corresponding D-forms as components of the nucleic acid (cf. WO 98/08856). In the context of the present invention, it was shown that HAS-nucleic acid conjugates can be prepared which may retain their natural function (cf. example 7).

The present invention further provides processes for the preparation of covalent HAS-active ingredient conjugates. The processes can be performed in an aqueous or organic reaction medium, wherein carrying out the coupling in an aqueous medium is preferred.

Thus, processes for the preparation of a covalent HAS-active ingredient conjugate are provided in which HAS and at least one active ingredient are reacted with each other in a reaction medium. The reaction medium is characterized in that it is water or a mixture of water with an organic solvent, which comprises at least 10 weight-% water.

The reaction medium of the process according to the invention comprises at least 10 wt.-%, preferably at least 50 wt.-%, in particular at least 80 wt.-%, such as for example 90 wt.-%, or



even up to 100 wt.-%, water, and accordingly up to 90 wt.-%, preferably up to 50 wt.-%, in particular up to 20 wt.-%, for example 10 wt.-%, or even up to 0 wt.-%, organic solvent. The reaction takes place in an aqueous phase. The preferred reaction medium is water.

The process according to the invention is already advantageous because toxicologically unacceptable solvents need not necessarily be used, and thus, with the product prepared according to the invention, the removal of even small residues of toxicologically unacceptable solvents which is always necessary according to the known process in order to avoid the undesired contamination with solvent is dispensed with. Furthermore, the additional quality control necessary according to the process known in the art for residues of toxicologically harmful solvents can be omitted because the process according to the invention favours the use of toxicologically acceptable solvents. Solvents preferred according to the invention are for example toxicologically harmless protic solvents such as ethanol or propylene glycol.

Furthermore, it is an advantage of the process according to the invention that irreversible or reversible structural changes of proteins or peptides induced by organic solvents, which cannot be systematically excluded in processes in organic solvents, are basically avoided. The product obtained with the process according to the invention is consequently different from that prepared in DMSO.

According to the invention it was, furthermore, surprisingly found that a coupling of HAS to active ingredients can be carried out in an aqueous solution without secondary reactions being observed to a significant extent. The process according to the invention thus leads directly to improved products of great

purity. The process according to the invention thus makes possible for the first time the simple preparation of HAS-active ingredient conjugates in which the active ingredient is present in active form and the advantageous properties of the HAS are retained. No particular processes are necessary to isolate the HAS-active ingredient conjugate from the reaction mixture as the reaction takes place in the aqueous phase, i.e. organic solvents need not necessarily be purified off.

According to the invention it is preferred that HAS binds directly to a  $\varepsilon$ -NH<sub>2</sub>-group,  $\alpha$ -NH<sub>2</sub>-group, SH-group, COOH group or -C(NH<sub>2</sub>)<sub>2</sub>-group of the active ingredient. Alternatively, a further reactive group can be introduced into HAS or the bond between HAS and the active ingredient can take place via a linker. The use of the corresponding linkers for the binding of active ingredients to PEG is known in the state of the art. The use of amino acids, in particular glycine, alanine, leucine, isoleucine, and phenylalanine, as well as hydrazine and oxylamine derivatives as linkers, as disclosed in WO 97/38727 and EP 605 963, is preferred.

According to one embodiment of the process of the present invention, HAS is oxidized before binding to the active ingredient. The oxidation can take place according to one of the processes known in the state of the art, a selective oxidation of the reducing end groups of HAS being preferred. This facilitates processes in which the oxidized reducing end group of the HAS reacts with an amino group of the active ingredient resulting in the formation of an amide. This embodiment has the particular advantage that a specific bond between HAS and the active ingredients, and thus a particularly homogeneous product, is achieved.

HAS can be reacted with oxidized reducing end groups and the

active ingredient preferably for at least 12, most preferably at least 24 hours. Furthermore, it can be desirable to add any activator, for example ethyldimethyl-aminopropyl-carbodiimide (EDC). The molar ratio between HAS and the active ingredient during the reaction can be randomly selected, but is normally in the range of HAS:active ingredient of 20:1 to 1:20, a ratio of 6:1 to 1:6 being particularly preferred. The best results were achieved with a molar ratio of HAS:active ingredient of approx. 2:1.

Other coupling reactions between an amino group of the active ingredient and HAS are naturally also comprised in the scope of the invention, for example, processes in which HAS and the active ingredient are reacted directly with each other, a Schiff's base forming between HAS and active ingredient as intermediate product. The azomethine group  $-\text{CH}=\text{N}-$  of the Schiff's base can then be reduced with formal addition of  $\langle 2\text{H} \rangle$  to a methyleneamine group  $-\text{CH}_2-\text{NH}-$ . For this reduction, a person skilled in the art can use a number of reduction agents known in the state of the art, reduction using  $\text{BH}_3$  is particularly preferred.

The HAS can be coupled with any group of the active ingredient. The coupling is preferably carried out such that the conjugate displays at least 50%, preferably at least 75% of the activity of the active ingredient before coupling, a retention of at least 95% of the activity being particularly preferred. The coupling reaction can naturally also be controlled such that the HAS is bound exclusively to one or more specific groups of the active ingredient, for example to lysine or cysteine residues of a peptide. Particular advantages result if HAS is bound to one or more specific groups of active ingredient via the oxidized reducing end groups, as homogenous HAS-active ingredient conjugates are obtained with a corresponding process.

According to a preferred embodiment of the process of the present invention, HAS and a protein or a peptide are used as starting substances for the reaction. Any therapeutic or diagnostic proteins of natural or recombinant origin can be used. A list of the active ingredients of recombinant preparation currently on the market is to be found in Pharma Business, July/August 2000, pages 45 to 60. The present invention comprises the preparation of HAS-active ingredient conjugates which comprise any one of the active ingredients named in this publication.

The preparation of conjugates using cytokines, for example interferons, interleukins, growth factors, enzymes, enzyme inhibitors, receptors, receptor fragments, insulin, factor VIII, factor IX, antibiotics (or anti-infectives), peptide antibiotics, viral coat proteins, haemoglobins, erythropoietin, albumins, hTPA, antibodies, antibody fragments, single-chain antibodies, DNA, RNA or a derivative thereof is particularly preferred. Particular advantages result when using recombinant proteins or peptides in the process according to the invention. As already stated, corresponding proteins can often not be used as active ingredients due to their properties being antigenic for humans. After coupling to HAS by the processes according to the invention, however, the immunogenicity of the recombinant proteins decreases, which allows the medical use on humans.

Furthermore, particular advantages result upon coupling of HAS to proteins having a short chain and smaller peptides. Currently, a large number of peptide libraries are being produced, for example, phage display libraries wherein short oligopeptides (for example 3 to 20mers) are expressed on the surface of phages. Furthermore, antibodies from a single polypeptide chain (so-called "single chain antibodies") are expressed in bacteria or on the surface of phages. These libraries are screened for specific active ingredient or binding activity. Hitherto, the therapeutic

---

and diagnostic use of corresponding peptide active ingredients or antibodies has however failed because these are very quickly excreted from the organism due to their small size (cf. Chapman et al., 1999, loc. cit.). With the process according to the invention, these peptides can advantageously be coupled to HAS and have an in vivo half-life which allows to use the same as an active ingredient.

As an alternative to the above embodiment, a hormone, a steroid hormone, a hormone derived from amino acids or a hormone derived from fatty acids can be used as active ingredient. In the specific case, it may be necessary to introduce an active group into the hormone before binding to HAS, for example by using a linker.

According to the invention, any physiologically compatible HES can be used as starting material. HES with an average molecular weight (weight average) of 1 to 300 kDa, in particular of 1 to 150 kDa is preferred. HES with an average molecular weight of 2 to 40 kD is particular preferred. HES preferably has a molar degree of substitution of 0.1 to 0.8 and a ratio of  $C_2:C_6$  substitution in the range of 2 to 20, in each case relative to the hydroxyethyl groups.

The invention furthermore relates to the HAS-active ingredient conjugates obtainable according to the above processes. These conjugates have advantageous properties, namely high activity of the active ingredient, low immunogenicity, long residence time in the body and excellent rheological properties, which increase the medicinal benefit of the conjugates.

Accordingly, the present invention likewise comprises processes for preparing a medicament or diagnostic in which a HAS-active ingredient conjugate is prepared according to one of the above

processes and mixed with a pharmaceutically compatible carrier, adjuvant or auxiliary known in the state of the art, as well as medicaments or diagnostics obtainable according to this process.

The medicinal use of the corresponding medicament depends on the type of active ingredient used. If, for example, haemoglobin is used as active ingredient, the conjugate can be used as an oxygen transport agent. If on the other hand, a cytokine is used as active ingredient for the preparation, the conjugate can for example be used in tumor therapy. The concentration of the conjugate to be used in each case in the medicament can be ascertained immediately in dose-effect tests by any average person skilled in the art.

The diagnostics can be used in vivo or in vitro to diagnose illnesses or disorders. If an antibody or antibody fragment is used as active ingredient, the conjugate is suitable, for example, for carrying out the ELISA detection processes customary in the state of the art.

In the examples, the materials described in the following were used:

Human serum albumin:	Sigma-Aldrich A3782
HES 130 kD:	Type 130/0.5, prepared from T91SEP (Fresenius Kabi)
Data:	$M_w$ : 130 000 $\pm$ 20 000 D
$M_n$ :	42 600 D
HES 10 kD:	Type HHH 4-2 (Fresenius Kabi)
Data:	$M_w$ : 9 800 D
$M_n$ :	3 695 D

EDC: Sigma-Aldrich no. 16.146-2  
(ethyl dimethyl aminopropyl carbodiimide)  
HOBT Sigma-Aldrich no. 15.726-0  
(1-hydroxy-3H-benzotriazolhydrate)

DIG glycan detection kit: Roche-Boehringer no. 1142 372

The following examples 1 to 6 describe the coupling of HSA and diaminobutane to HES with oxidized reducing end groups or the direct coupling of HSA to HES. HSA and diaminobutane are simply examples of the active ingredients defined above. Example 7 describes the coupling of oligonucleotides to HES.

Example 1: Selective oxidation of the reducing end groups of the hydroxyethyl starch:

For the selective oxidation of the reducing end groups of the hydroxyethyl starch (130 kD and 10 kD), the same were dissolved in a minimal quantity of water and reacted with different quantities of an iodine solution and a KOH solution.

The mixture was stirred until the colour indicating  $I_2$  disappeared. This procedure was repeated several times in order to achieve the addition of a larger quantity of the iodine solution and KOH solution. The solution was subsequently purified using an Amberlite IR 120  $Na^+$  cation-exchanger resin, dialysed for 20 hours against distilled water (dialysis tube with an exclusion limit of 4-6 kD) and lyophilized.

The degree of oxidation was determined in each case using the process disclosed in Somogyi, N. (Method in Carbohydride Chemistry, Vol. 1, (1962) p. 384-386). The protocols of the

oxidation reaction are reproduced in Table 1.

Process	HES (Mn)	Iodine solution 0.1 N	KOH solution 0.1 N	Solvent	Reaction time	Yield
OXIDATION (1) HES 130	1 g $2.4 \times 10^{-5}$ mol	0.3 ml $3.0 \times 10^{-5}$ mol	0.5 ml $5.0 \times 10^{-6}$ mol	Water 4.0 ml	4 hours 25°C	30.1%
OXIDATION (2) HES 130	4 g $9.4 \times 10^{-6}$ mol	1.0 ml $1.0 \times 10^{-4}$ mol	1.5 ml $1.5 \times 10^{-4}$ mol	Water 6.0 ml	overnight 25°C	24.8%
OXIDATION (3) HES 130	5 g $1.2 \times 10^{-4}$ mol	1.2 ml $1.2 \times 10^{-4}$ mol	1.5 ml $1.5 \times 10^{-4}$ mol	Water 7.5 ml	overnight 25°C	24.3%
OXIDATION (4) HES 130	5 g $1.2 \times 10^{-4}$ mol	3.0 ml $3.0 \times 10^{-4}$ mol	4.5 ml $4.5 \times 10^{-4}$ mol	Water 7.5 ml	overnight 25°C	60.8%
OXIDATION (5) HES 130	5 g $1.2 \times 10^{-4}$ mol	4.0 ml $4.0 \times 10^{-4}$ mol	5 ml $5.0 \times 10^{-4}$ mol	Water 7.5 ml	overnight 25°C	80.0%
OXIDATION (6) HES 130	8 g $1.9 \times 10^{-4}$ mol	7.0 ml $7.0 \times 10^{-4}$ mol	11.5 ml $1.2 \times 10^{-3}$ mol	Water 7.5 ml	overnight 25°C	88.4%
OXIDATION (7) HES 130	10 g $2.4 \times 10^{-4}$ mol	10 ml $1.0 \times 10^{-3}$ mol	20 ml $2.0 \times 10^{-3}$ mol	Water 7.5 ml	overnight 25°C	100%
OXIDATION (1) HES 10	5 g $1.4 \times 10^{-3}$ mol	2.0 ml $2.0 \times 10^{-4}$ mol	2.0 ml $2.0 \times 10^{-4}$ mol	Water 10.0 ml	20 hours 25°C	3.0%
OXIDATION (2) HES 10	5 g $1.4 \times 10^{-3}$ mol	3.5 ml $3.5 \times 10^{-4}$ mol	4.5 ml $4.5 \times 10^{-4}$ mol	Water 10.0 ml	overnight 25°C	5.3%
OXIDATION (3) HES 10	15 g $4.1 \times 10^{-3}$ mol	21.0 ml $2.1 \times 10^{-3}$ mol	31.0 ml $3.1 \times 10^{-3}$ mol	Water	overnight 25°C	10.5%
OXIDATION (4) HES 10	8 g $2.2 \times 10^{-3}$ mol	83.0 ml $8.3 \times 10^{-3}$ mol	180.0 ml $1.8 \times 10^{-2}$ mol	Water	overnight 25°C	80.0%
OXIDATION (5) HES 10	7 g $1.3 \times 10^{-3}$ mol	95.0 ml $9.5 \times 10^{-3}$ mol	210.0 ml $2.1 \times 10^{-2}$ mol	Water	overnight 25°C	100.0%
OXIDATION (6) HES 10	6.4 g $1.7 \times 10^{-3}$ mol	50 ml $5.0 \times 10^{-3}$	150 ml $1.5 \times 10^{-2}$ mol	Water	overnight 25°C	100.0%

Table 1: Oxidation of the reducing end groups of the HES (130 kD and 10kD) under different conditions



The protocols summarized in this table are reproduced in detail in the following for the oxidation (6), HES 10 kD: 6.4 g HES 10kD (1.7 mmol) were dissolved in a reaction vessel accompanied by continuous stirring in a few ml water. 50 ml of a 0.1 N iodine solution (5.0 mmol) and 150 ml of a 0.1 N KOH solution (15 mmol) were added. This mixture was left to stand overnight at 25°C. The mix was purified with Amberlite IR 120 (Na<sup>+</sup> form) and dialysed against water (cellulose acetate dialysis tube; Cut-off 4 to 6 kD). The dialysed product was lyophilized (Heraeus-Christ Alpha, flask-drying overnight).

As can be inferred from Table 1, a complete oxidation of the reducing end groups (corresponds to a yield of 100%) of the HES 130 kD was achieved, after the iodine quantity was increased from  $3.0 \times 10^{-5}$  mol to  $1.0 \times 10^{-3}$  ml.

For a complete oxidation of the reducing end groups of the HES 10kD, a further increase of the iodine quantity to a concentration of more than  $2.1 \times 10^{-3}$  was necessary.

Example 2: Binding of HES with oxidized reducing end groups to HSA in the aqueous phase:

For the coupling, hydroxyethyl starch with oxidized reducing end groups (ox-HES) and HSA were completely dissolved in water. When the solution was clear, EDC dissolved in water was added. After activation by EDC accompanied by moderate stirring, further quantities of EDC were added. Where appropriate, the reaction was activated with HOBt and left to stand overnight. The product was purified for 15 hours by dialysis against distilled water and subsequently lyophilized (called Process A in the following).

The protocols of the coupling reaction are in Table 2.

Process A	HSA	ox-HES (Mn)	EDC	HOBt	Solvent	Activation	Reaction
Coupling I ox-HES 130	300 mg 4.4x10 <sup>-6</sup> mol	100 mg (1) 2.4x10 <sup>-6</sup> mol	25 mg 1.6x10 <sup>-4</sup> mol	100 mg 7.7x10 <sup>-4</sup> mol	H <sub>2</sub> O/dioxane 13 ml/2 ml	1.5 hours 3-4°C	4 hours 25°C
Coupling II ox-HES 130	100 mg 1.5x10 <sup>-6</sup> mol	300 mg (2) 7.0x10 <sup>-6</sup> mol	15 mg 9.7x10 <sup>-5</sup> mol	100 mg 7.7x10 <sup>-4</sup> mol	H <sub>2</sub> O/dioxane 10 ml/3 ml	1.5 hours 3-4°C	overnight 25°C
Coupling III ox-HES 130	200 mg 3.0x10 <sup>-6</sup> mol	3.8 g (5) 8.9x10 <sup>-5</sup> mol	46.5 mg 3.0x10 <sup>-4</sup> mol	20 mg 1.5x10 <sup>-4</sup> mol	H <sub>2</sub> O/dioxane 10 ml/3 ml	0 hours	24 hours 25°C
Coupling IV ox-HES 130	100 mg 1.5x10 <sup>-6</sup> mol	1.9 g (5) 4.5x10 <sup>-5</sup> mol	25 mg 1.6x10 <sup>-4</sup> mol	20 mg 1.5x10 <sup>-4</sup> mol	Water	1.5 hours 3-4°C	overnight 25°C
Coupling V ox-HES 130	200 mg 3.0x10 <sup>-6</sup> mol	4.3 g (5) 1.0x10 <sup>-4</sup> mol	100 mg 6.0x10 <sup>-4</sup> mol	0 mg	Water	0 hours	overnight 25°C
Coupling VI ox-HES 130	100 mg 1.5x10 <sup>-6</sup>	130 mg (7) 3.0x10 <sup>-5</sup> mol	50 mg 3.0x10 <sup>-4</sup> mol	0 mg	Water* 5 ml+10 ml	0 hours	5 hours 25°C
Coupling VII ox-HES 10	100 mg 1.5x10 <sup>-6</sup>	130 mg (7) 3.0x10 <sup>-5</sup> mol	200 mg 3.0x10 <sup>-4</sup> mol	0 mg	Water* 5 ml+2x10 ml	0 hours	24 hours 25°C
Coupling I ox-HES 10	100 mg 1.5x10 <sup>-6</sup> mol	300 mg (1) 8.1x10 <sup>-5</sup> mol	5 mg 3.0x10 <sup>-5</sup> mol	100 mg 7.7x10 <sup>-4</sup> mol	H <sub>2</sub> O/dioxane 13 ml/2 ml	1.5 hours 3-4°C	overnight 25°C
Coupling II ox-HES 10	70 mg 1.0x10 <sup>-6</sup> mol	1.0 g (2) 2.7x10 <sup>-4</sup> mol	15.5 mg 1.0x10 <sup>-4</sup> mol	0 mg	Water	10 ml 0 hours	overnight 25°C
Coupling III ox-HES 10	200 mg 3.0x10 <sup>-6</sup> mol	2.0 g (3) 8.1x10 <sup>-4</sup> mol	77.5 mg 5.0x10 <sup>-4</sup> mol	20 mg 1.5x10 <sup>-4</sup> mol	Water	0 hours	6 hours 25°C
Coupling IV ox-HES 10	50 mg 7.4x10 <sup>-7</sup> mol	7.4 g (4) 2.0x10 <sup>-3</sup> mol	282 mg 1.5x10 <sup>-3</sup> mol	0 mg	Water	0 hours	overnight 25°C
Coupling V ox- HES 10	100 mg 1.5x10 <sup>-6</sup> mol	103 g (6) 2.8x10 <sup>-5</sup> mol	93 mg 5.6x10 <sup>-4</sup> mol	0 mg	Water* 4 ml	0 hours	20 hours 25°C
Coupling VI ox-HES 10	100 mg 1.5x10 <sup>-6</sup> mol	103 g (6) 2.8x10 <sup>-5</sup> mol	200 mg 1.2x10 <sup>-3</sup> mol	0 mg	Water* 3x5 ml	0 hours	30 hours 25°C

\*= Addition of the EDC solution with a dropping funnel within  
60 minutes

Table 2: Coupling reactions between HES (130 kD and 10 kD) with oxidized reducing end groups and HSA under different conditions (Process A; number in brackets in the HES column reproduces the oxidation process according to Table 1).

The coupling reaction VII between ox-HES 130 kD and HSA is explained in detail in the following: In a round-bottomed flask, 130 mg ox-HES 130 kD (degree of oxidation approx. 100%) and 100 mg HSA were dissolved accompanied by stirring in approx. 5 ml water at room temperature. As soon as the solution was clear, 200 mg EDC in 3 portions, each dissolved in 5-10 ml water, were added over a period of one hour. Between each addition, the reaction mixture was stirred at room temperature for about 4 hours. After 24 hours reaction time, the mixture was dialysed against water (cellulose acetate dialysis tube; Cut-off 4 to 6 kD). The product was then lyophilized.

Example 3: Direct binding of HES to HSA in the aqueous phase:

The principle of this reaction is based on the formation of Schiff's bases between HES and amino groups of the protein, the reaction being controlled through the reaction of the Schiff's bases to the corresponding amine by  $\text{NaBH}_4$  (called Process B in the following).

For this, HES was dissolved completely in a small amount of water. To this end, HSA dissolved in borate buffer, pH 9.0, was added.  $\text{NaBH}_4$  was added to this solution and the whole left at room temperature accompanied by stirring. A further aliquot of HES 130 kD, followed by further  $\text{NaBH}_4$ , was added. After the reaction was finished, dialysis and freeze-drying was carried out

as described.

The protocols of the individual tests are summarized in Table 3.

Process B	HSA	HES (Mn)	NaBH <sub>4</sub>	Buffer pH	Reaction time
COUPLING I	50 mg	500 mg	500 mg	Na <sub>2</sub> HPO <sub>4</sub> , 0 ml	48 hours
HES 130	$7.5 \times 10^{-7}$ mol	$1.2 \times 10^{-5}$ mol	$1.3 \times 10^{-2}$ mol	7.4	25°C
COUPLING II	100 mg	1.0 g	60 mg	Na <sub>2</sub> HPO <sub>4</sub> , 1 ml	20 hours
HES 130	$1.5 \times 10^{-6}$ mol	$2.4 \times 10^{-5}$ mol	$1.6 \times 10^{-3}$ mol	7.4	25°C
COUPLING III	50 mg	9.8 g	285 mg	Na <sub>2</sub> HPO <sub>4</sub> , 1 ml	36 hours
HES 130	$7.5 \times 10^{-7}$ mol	$2.3 \times 10^{-4}$ mol	$7.5 \times 10^{-3}$ mol	7.4	25°C
COUPLING IV	50 mg	2.0 g	180 mg	Borate 0.1M	30 hours
HES 130	$7.5 \times 10^{-7}$ mol	$4.7 \times 10^{-5}$ mol	$4.7 \times 10^{-3}$ mol	7.4	25°C
COUPLING V	50 mg	4.0 g	60 mg	Borate 0.1M	100 hours
HES 130	$7.5 \times 10^{-7}$ mol	$9.4 \times 10^{-5}$ mol	$1.6 \times 10^{-3}$ mol	7.4	25°C
COUPLING I	50 mg	2.8 g	28 mg	Borate 0.1M	80 hours
HES 10	$7.5 \times 10^{-7}$ mol	$9.4 \times 10^{-5}$ mol	$1.6 \times 10^{-3}$ mol	9.0	25°C

Table 3: Direct coupling between HES (130 kD and 10 kD) and HSA under different conditions (Process B).

In detail, for the coupling of the HES 130 kD, 2.0 g of this compound were completely dissolved in water (approx. 5 ml). 50 mg of HSA dissolved in 1 ml 0.1 M borate buffer, pH 9.0 were added. 30 mg NaBH<sub>4</sub> were added to this solution and the whole left at room temperature accompanied by stirring. After 18 h, a further aliquot of 2.0 g HES 130 kD, followed by a further 30 mg NaBH<sub>4</sub>, were added. After 100 h reaction time in total, dialysis and freeze-drying were carried out (coupling V, HES 130 kD).

For the coupling of the HES 10 kD, 1.4 g of this compound were completely dissolved in water (approx. 5 ml). 50 mg of HSA dissolved in 1 ml 0.1 M borate buffer, pH 9.0, were added. 14 mg  $\text{NaBH}_4$  were added to this solution and the whole left at room temperature accompanied by stirring. After 18 hours a further aliquot of 1.4 g HES 10 kD, followed by a further 14 mg  $\text{NaBH}_4$ , was added. After a total of 80 hours reaction time, dialysis and freeze-drying were carried out (coupling I, HES 10 kD).

Example 4: Analysis of the coupling products using GPC:

The reaction products were first analyzed using gel-permeation chromatography (GPC).

4.1 An FPLC device (Pharmacia) which was connected to a HPLC UV monitor (Hitachi) was used for the GPC. Furthermore, the following conditions were used:

Column:	Superose 12 HR 10/30 (1x30 cm) (Pharmacia)
UV monitor:	280 nm
Pump:	0.2 ml/min
Buffer:	50 mM phosphate/150 mM NaCl pH 7.2.

Under these conditions, the HSA peak is normally found after 63 minutes, a small peak, which is caused by HSA dimers, also being able to be measured at approx. 57 minutes. The

chromatograms obtained by means of GPC can be analyzed as follows:

- 4.2 Fig. 1 is a chromatogram which shows the size distribution of the products after coupling of ox-HES 130 kD to HSA (coupling III). With this coupling process, very good results were achieved without HOBt activation. A clear, single broad peak was measured at 37 minutes and a further, smaller band at 45 minutes, which indicates a coupling product with higher molecular weight than HSA. At the same time, traces of non-modified HSA were found.

Fig. 2 shows the size distribution of the products after coupling of ox-HES 130 kD to HSA (coupling IV). The reaction was activated with HOBt. It is shown that this activation reduces the yield, possibly by encouraging secondary reactions.

Figs. 3 and 4 show the size distribution of the reaction products during and after the coupling reaction of ox-HES 130 kD to HSA (coupling V). After 2 hours reaction time, non-modified HSA was found as the product with the highest concentration, but in addition the first coupling products with a higher molecular weight were found. After the reaction was finished, a homogeneous coupling product with a retention time of approx. 35 minutes was found in high concentration. Non-modified HSA and other coupling products were present in relatively low concentration.

Fig. 5 shows the corresponding size distribution of the reaction products during and after the coupling reaction of ox-HES 10 kD to HSA (coupling V). Here too, it is shown that the concentration of the coupling products with a molecular

weight which lies above the weight of HSA increases in the course of the reaction.

Finally, a coupling reaction in which almost all HSA molecules were able to be transferred into a homogenous coupling product is shown in Fig. 6 (reaction products of coupling VII).

- 4.2 An example of the chromatograms which were obtained upon analysis of the direct coupling of HES to HSA is shown in Fig. 7 (Process B, HES 130 kD, coupling V). A significant peak was identified at approx. 65 minutes (HSA). In addition, however, a coupling product was also shown (peak at approx. 42 minutes).

Example 5: Analysis of the coupling products by means of SDS-PAGE and Western Blot:

- 5.1 PAGE was carried out in the presence of sodium dodecyl sulfate (SDS) using a Miniprotean II device from Biorad and 7.5% acrylamide gels. Electrophoresis was carried out as per the manufacturers instructions. The gels were stained with silver using the Blum process (Elektrophoresis, Vol. 8, (1997) p. 93-99), to make proteins visible.

The presence of glycans in the coupling products was detected by means of Western-Blot and Glycan-Detections-Kit from Roche-Boehringer. After separation of the products by means of SDS-PAGE, these were transferred to a nitrocellulose membrane using the blotting apparatus of the Miniprotean II electrophoresis unit. The membrane was then oxidized using periodate under conditions in which only the

vicinal OH groups are oxidized. These were then reacted with an amino-functionalized digoxigenin. Bound digoxigenin was detected by means of specific monoclonal antibodies which were bound to an alkaline phosphatase. For this, a substrate of the phosphatase (4-nitro-tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate) was added, which produces a difficultly soluble blue-violet product. This product precipitates onto the membrane and thus renders the bands visible. Non-modified HSA and creatinase were used as negative controls whilst transferrin served as positive control.

The exact process steps are described in the instruction leaflet enclosed with this kit (Roche-Boehringer).

5.2 Figs. 8 and 9 each show a picture of the silver-stained SDS-PAGE gel (top) and the picture of the corresponding products after transfer onto a membrane and glycan detection (bottom). As can be inferred from these figures, a relatively homogeneous glycan forms as a reaction product during the coupling reaction, whilst at the same time the concentration of non-modified HSA decreases.

1

Example 6:      Analysis of potential secondary reactions:

To determine whether secondary reactions in the form of a self-condensation of HES with oxidized reducing end groups occur, the following reaction mixtures were prepared:



	$\alpha$ -HES	EDC	HOBt	Water	Reaction time
HES 130 kD	500 mg $1.2 \times 10^{-5}$ mol	15 mg $7.8 \times 10^{-5}$ mol	-	5.0 ml	30 hours 25°C
HES 130 kD	500 mg $1.2 \times 10^{-5}$ mol	15 mg $7.8 \times 10^{-5}$ mol	Saturated solution	5.0 ml	30 hours 25°C
HES 10 kD	100 mg $2.7 \times 10^{-5}$ mol	3.4 mg $1.8 \times 10^{-5}$ mol	-	5.0 ml	30 hours 25°C
HES 10 kD	100 mg $2.7 \times 10^{-5}$ mol	3.4 mg $1.8 \times 10^{-5}$ mol	saturated solution	5.0 ml	30 hours 25°C
HES 130 kD	700 mg $1.6 \times 10^{-5}$ mol	31 mg $1.6 \times 10^{-4}$ mol	-	5.0 ml	30 hours 25°C
HES 130 kD	700 mg $1.6 \times 10^{-5}$ mol	31 mg $1.6 \times 10^{-4}$ mol	Saturated solution	5.0 ml	30 hours 25°C

Table 4: Reaction mixtures for the analysis of secondary reactions

The aim of the experiments was to demonstrate the extent to which a potential self-condensation of HES takes place in the presence or absence of HOBt. The samples were lyophilized and forwarded to Fresenius-Kabi for the carrying out of the analysis.

By means of GPC and light-scatter measurements, within the detection limits of a few per cent, no indications of increases in molecular weight were found.

Example 7: Coupling of oxidized HES to DNA and analysis of the functionality of the coupling products

**Reaction principle:**

A schematic representation of the reaction can be found in Figure 10. In a first step, the amino group of the amine-HES12KD 3 reacts with the N-hydroxysuccinimid group of SMCC 4 to form conjugate 5. SMCC 4 which has not reacted is separated by centrifugation using a centrifugation/dialysis unit. The maleimid-group of conjugate 5 subsequently reacts with the thiol group of the thio-DNA 1 to the desired product 6. The region in 6, which is marked in bold mererly represents a spacer and can have any form.

Evaluation of the biological activity of conjugate 6 was performed via restriction with the restriction enzyme EcoR1. Restriction enzymes only cleave double-stranded DNA with an intact recognition sequence.

**DNA:**

Double-stranded DNA synthesized by MWG Biotech Corporation, Ebersberg, Germany, was used. The sequences of the single strands are:

SEQ ID NO. 1:

5' -GTAGAGACAGGAGGCAGCAGTTGAATTGCGAGGGTGAGTAGCAGTAGAGC-3' ;

SEQ ID NO: 2:

5'-GCTCTACTGCTACTCACCCCTGCGAATTCAACTGCTGCCTCCTGTCTCTAC-3';

modified with 5'thiol C6 S-S by MWG (cf. Figure 10).

Both DNA single strands were dissolved in bidest. Water in a concentration of 2  $\mu\text{g}/\mu\text{l}$ , and were hybridized in a ratio of 1:1 at 96°C to a double-stranded thio-DNA 1 with a concentration of 2  $\mu\text{g}/\mu\text{l}$ .

#### Analysis of the products:

Analysis was performed by a gel electrophoresis on a 4 % agarose gel with a TBE running buffer consisting of 45 mM Tris borate, 1 mM EDTA, pH 8.0, using 1  $\mu\text{g}$  DNA in the presence of 50  $\mu\text{g}$  ethidium bromide per 100 ml gel in each case. The images were taken by use of a CCD-system modular (INTAS Imaging Instruments, Göttingen, Germany) and an UV transilluminator UVT-20 S/M/L (Herolab, Wiesloch, Germany) at 312 nm.

1  $\mu\text{l}$  (1  $\mu\text{g}$  DNA) of the reaction mix were taken and cleaved with 1  $\mu\text{l}$  (20 U) EcoRI restriction enzyme (New England Biolabs, Schwalmbach/Taunus, Germany), 1  $\mu\text{l}$  reaction buffer (50 mM sodium chloride, 100 mM Tris HCl, 10 mM magnesium chloride, 0.025 % Triton X-100, pH 7,5 from New England Biolabs) and 7  $\mu\text{l}$  bidest. water at 37°C for 3 hours.

#### Modification of HES

Oxidation of HES12KD (Fresenius, Lot 2540SR2.5P) with an average molecular weight of 12.000 g/mol with iodine solution to oxo-HES12KD 2 was performed according to the process disclosed in DE 196 28 705.

Reaction of 1,4-diaminobutane with oxo-HES12KD 2:

1.44 g (0.12 mmol) of oxo-HES12KD 2 were dissolved in water-free dimethyl sulfoxide (DMSO), added dropwise to a solution of 1.5 ml (1.50 mmol) -1,4 diaminobutane under nitrogen, and stirred at 40°C for 19 hours. The reaction mixture was added to a mixture of 80 ml ethanol and 80 ml acetone. The precipitate formed was separated by centrifugation and resuspended in 40 ml water. The solution was dialyzed for 4 days against water (SnakeSkin dialysis tube, 3.5 KD cut off, Perbio Science Deutschland, Bonn, Germany) and subsequently lyophilized. The yield was 80 % (1.06 g) amino-HES12KD 3.

#### Coupling of amino-HES12KD 3 to thio-DNA 1.

1 mg SMCC 4 dissolved in 50  $\mu$ l water-free DMSO were added to 400  $\mu$ l of a 10 mg/ml-solution of amino-HES12KD 3 in a buffer of 10 mM sodium phosphate and 150 mM sodium chloride, pH 7.44, and the mixture was incubated 80 min at room temperature and 10 min at 46°C. Subsequently, the mixture was centrifuged, the supernatant was removed from the precipitate, and it was again centrifuged. 200  $\mu$ l of the supernatant were taken and centrifuged for 45 minutes at 14000 g with a MICROCON YM-3 (Amicon, Millipore, Eschborn, Germany) centrifugation-dialysis unit. After the addition of 400  $\mu$ l buffer consisting of 10 mM sodium phosphate and 150 mM sodium chloride, pH 7.44, it was again centrifuged for 45 minutes. Additional 400  $\mu$ l buffer were added, and it was centrifuged for 60 minutes. The amount of conjugate solution which was left in the dialysis unit was filled up to 50  $\mu$ l. 10  $\mu$ l of this solution were added to 10  $\mu$ l of thio-DNA solution 1 and both were reacted with each other for 14 hours at room temperature. 1  $\mu$ l was taken for analysis. Figure 11 shows the results in lanes 2 and 3.

The reaction conditions for the described experiment and for experiments with modified reaction conditions are summarized in Table 1. The results are depicted in Figure 11.

Summary of the results:

The following conditions were analyzed:

1. Amount of SMCC: 1 mg (lanes 2, 6, 10, 14) or 5.6 mg (lanes 4, 8, 12, 16), respectively;
2. Temperature for the reaction with thio-DNA 1: room temperature (lanes 2, 4, 6, 8) or 37°C (lanes 10, 12, 14, 16), respectively;
3. Buffer conditions:  
  
10 mM phosphate, 150 mM sodium chloride without EDTA, pH 7.44 (lanes 2, 4, 10, 12) or 100 mM phosphate, 150 mM sodium chloride + 50 mM EDTA, pH 7.23 (lanes 6, 8, 14, 16), respectively.

In Figure 11, lanes 2-18 represent the results of the 8 coupling experiments of amino-HES12KD 3 to thio-DNA 1 using SMCC. The results directly from the reaction or after the reaction and subsequent digestion of the DNA, respectively, are depicted next to each other. In lane 1, a mixture of different reference DNAs is loaded as length marker. Lanes 18 and 19 show thio-DNA 1 or digested thio-DNA 1, respectively. In addition to non-reacted thio-DNA 1, all experiments show coupling products at higher masses (lanes 2, 4, 6, 8, 10, 12, 14, 16). Since HES12KD is a mixture of molecules of different size, the coupling products also show a molecular weight distribution. All coupling products contain intact DNA as they can be completely digested by EcoR1.

This is also demonstrated by nearly complete vanishing of corresponding diffuse bands after digestion (lanes 3, 5, 7, 9, 11, 13, 15, 17).

Table 1: Reaction conditions of the coupling of amino-HES12KD 3 to thio-DNA 1

Lane	Experiment	Temp. [°C]	SMCC [mg]	DNA	HES12KD	Buffer	
1	Marker						
2	19A1	RT	1	thio-DNA	amino-HES12KD	7.44, 10 mM	
3							digested
4	19B1	RT	5.6	thio-DNA	amino-HES12KD	7.44, 10 mM	
5							digested
6	19C1	RT	1	thio-DNA	amino-HES12KD	7.23, 100 mM	
7							digested
8	19D1	RT	5.6	thio-DNA	amino-HES12KD	7.23, 100 mM	
9							digested
10	19A2	37°C	1	thio-DNA	amino-HES12KD	7.44, 10 mM	
11							digested
12	19B2	37°C	5.6	thio-DNA	amino-HES12KD	7.44, 10 mM	
13							digested
14	19C2	37°C	1	thio-DNA	amino-HES12KD	7.23, 100 mM	
15							digested
16	19D2	37°C	5.6	thio-DNA	amino-HES12KD	7.23, 100 mM	
17							digested
18				thio-DNA			
19							digested

1. Process for preparation of a covalent HAS-active ingredient conjugate wherein HAS and an active ingredient are coupled in a reaction medium, wherein
  - (a) the reducing end groups of the HAS are selectively oxidized prior to binding to the active ingredient;
  - (b) the reaction medium is water or a mixture of water with an organic solvent which comprises at least 10 weight-% water; and
  - (c) the active ingredient is a protein, oligo- or polypeptide.
2. Process according to claim 1, wherein the active ingredient is a vaccine, toxin, antibiotic (anti-infective), antiarrhythmic, appetite suppressant, anesthetic, analgesic, antirheumatic, antiallergic, antiasthmatic, antidepressant, antidiabetic, antihistamine, antihypertonic or an antineoplastic agent.
3. Process according to claim 1 or 2, wherein the active ingredient is an enzyme, enzyme inhibitor, receptor, receptor fragment, insulin, factor VIII, factor IX, cytokine, interferon, interleukin, growth factor, peptide antibiotic, viral coat protein, haemoglobin, erythropoietin, albumin, hTPA, antibody, antibody fragment, or a single-chain antibody.
4. A process according to one of the preceding claims, wherein HAS binds to the  $\epsilon$ -NH<sub>2</sub>-group, to the  $\alpha$ -NH<sub>2</sub>-group, to the SH group, to a COOH group or to a -C(NH<sub>2</sub>)<sub>2</sub>-group of the active ingredient.

5. Process according to one of the preceding claims, wherein the oxidized reducing end group of the HAS reacts with an amino group of the active ingredient resulting in the formation of an amide.
6. Process according to one of the preceding claims, wherein the active ingredient or HAS is bound to a linker before the preparation of the conjugate.
7. Process to one of the preceding claims, wherein hydroxyethyl starch with an average molecular weight (weight average) of 1 to 300 kDa is used.
8. Process according to one of the preceding claims, wherein hydroxyethyl starch with an average molecular weight of 2 to 40 kDa is used.
9. Process according to one of the preceding claims, wherein hydroxyethyl starch with a molar degree of substitution of 0.1 to 0.8 and a ratio of C<sub>2</sub>:C<sub>6</sub> substitution in the range from 2 to 20, in each case relative to the hydroxyethyl groups, is used.
10. Process for the preparation of a medicament or diagnostic, comprising steps in which a HAS-active ingredient conjugate is prepared according to one of claims 1 to 9, and is mixed a pharmaceutically compatible carrier, adjuvant or auxiliary compound.



DRW - 14.

Application number / numéro de demande: EP02/02928

Figures: 8, 9, 11

Pages: \_\_\_\_\_

Unscannable items  
received with this application  
(Request original documents in File Prep. Section on the 10<sup>th</sup> floor)

Documents reçu avec cette demande ne pouvant être balayés  
(Commander les documents originaux dans la section de préparation des dossiers au  
10ème étage)

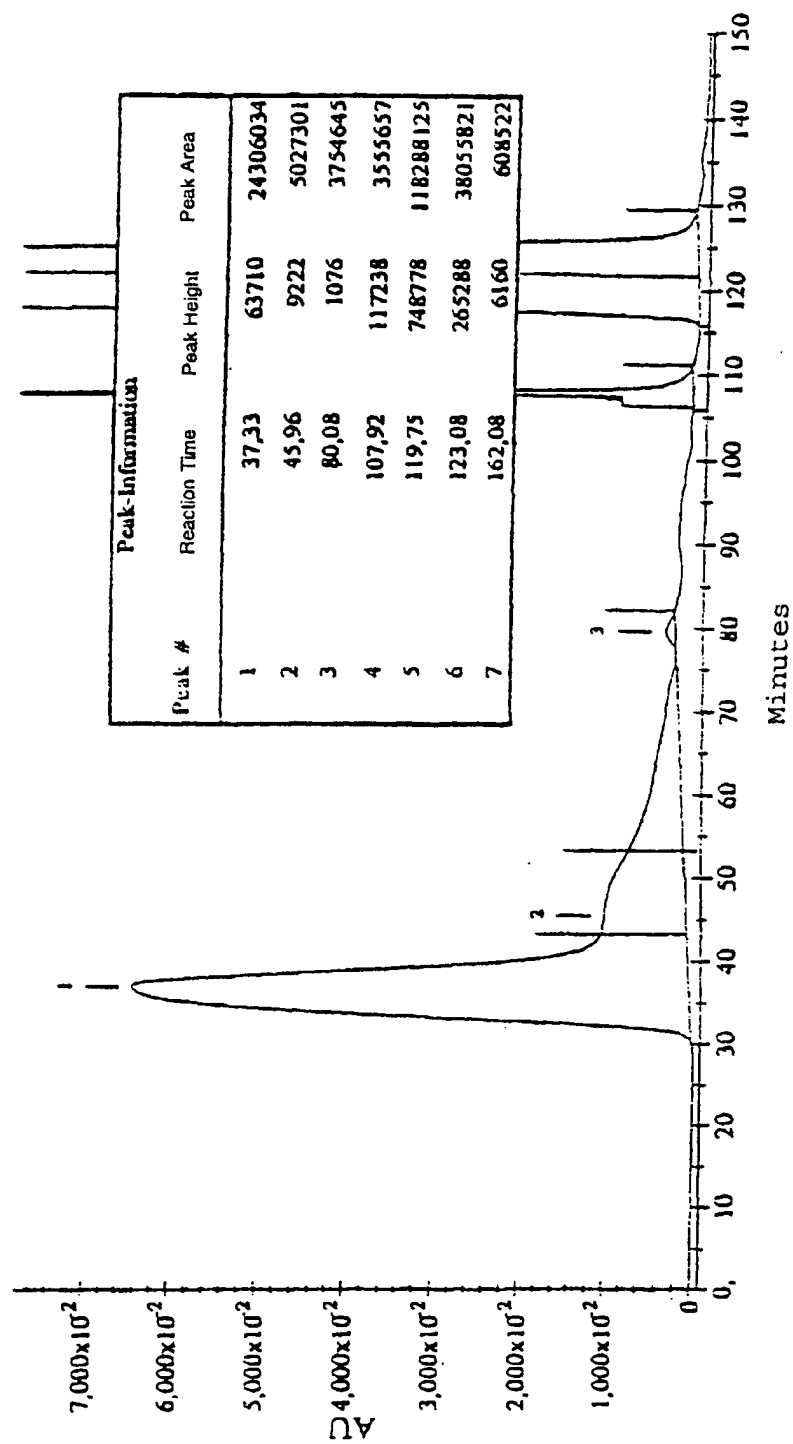


Fig. 1: GPC chromatogram of coupling III ox-HES 130 kD to HSA

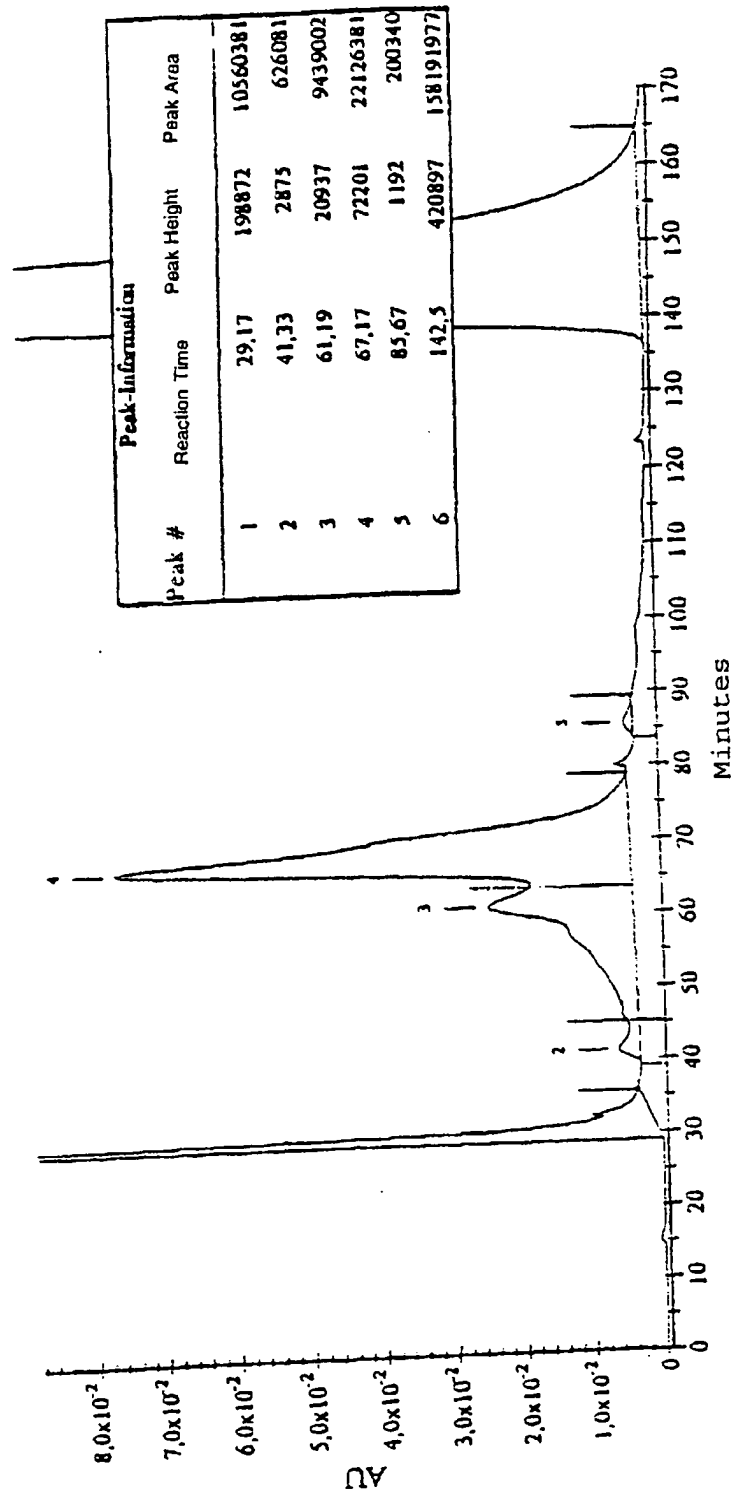


Fig. 2: GPC chromatogram of coupling IV ox-HES 130 kD to HSA

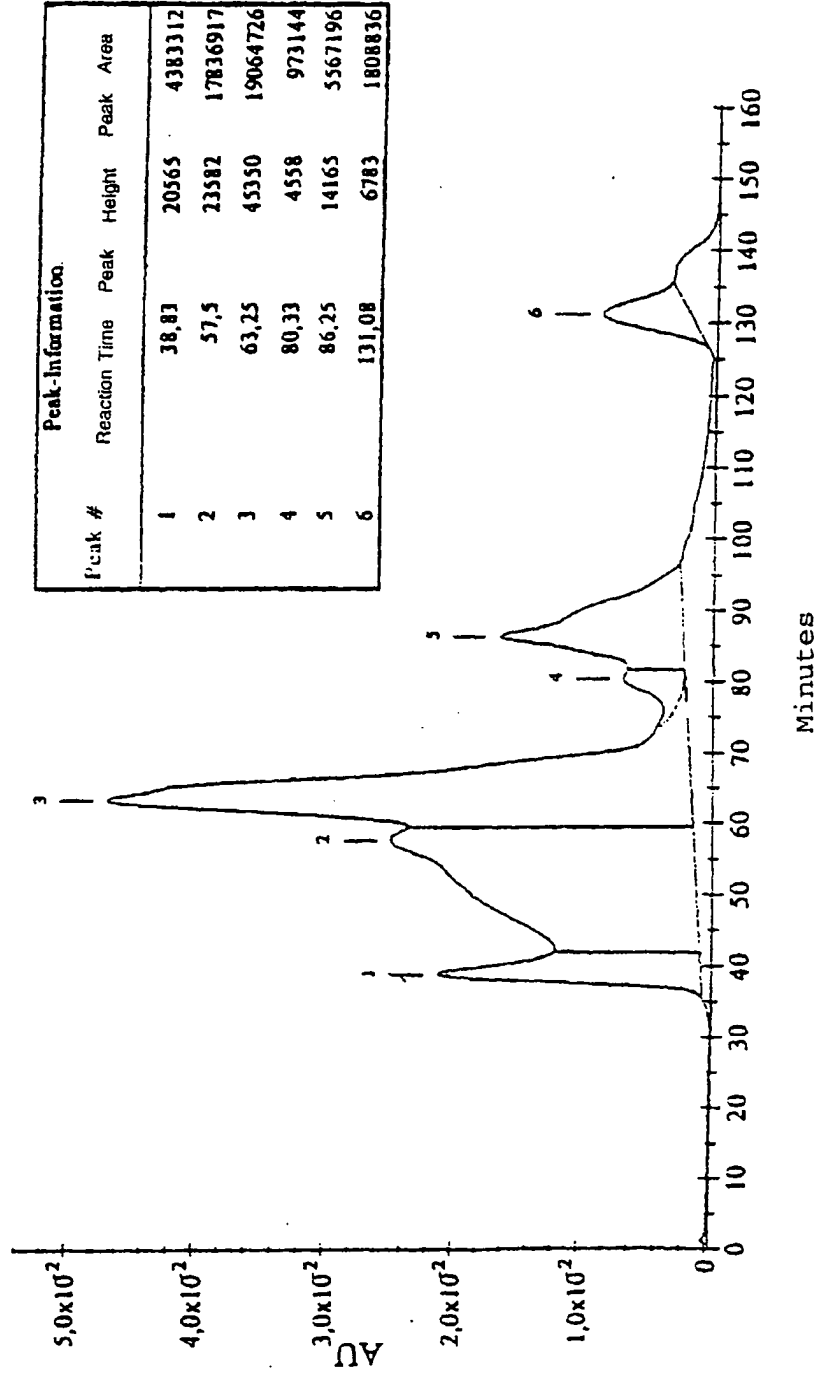


Fig. 3: GPC chromatogram of coupling V ox-HES 130 kD to HSA (reaction time 2 hours)

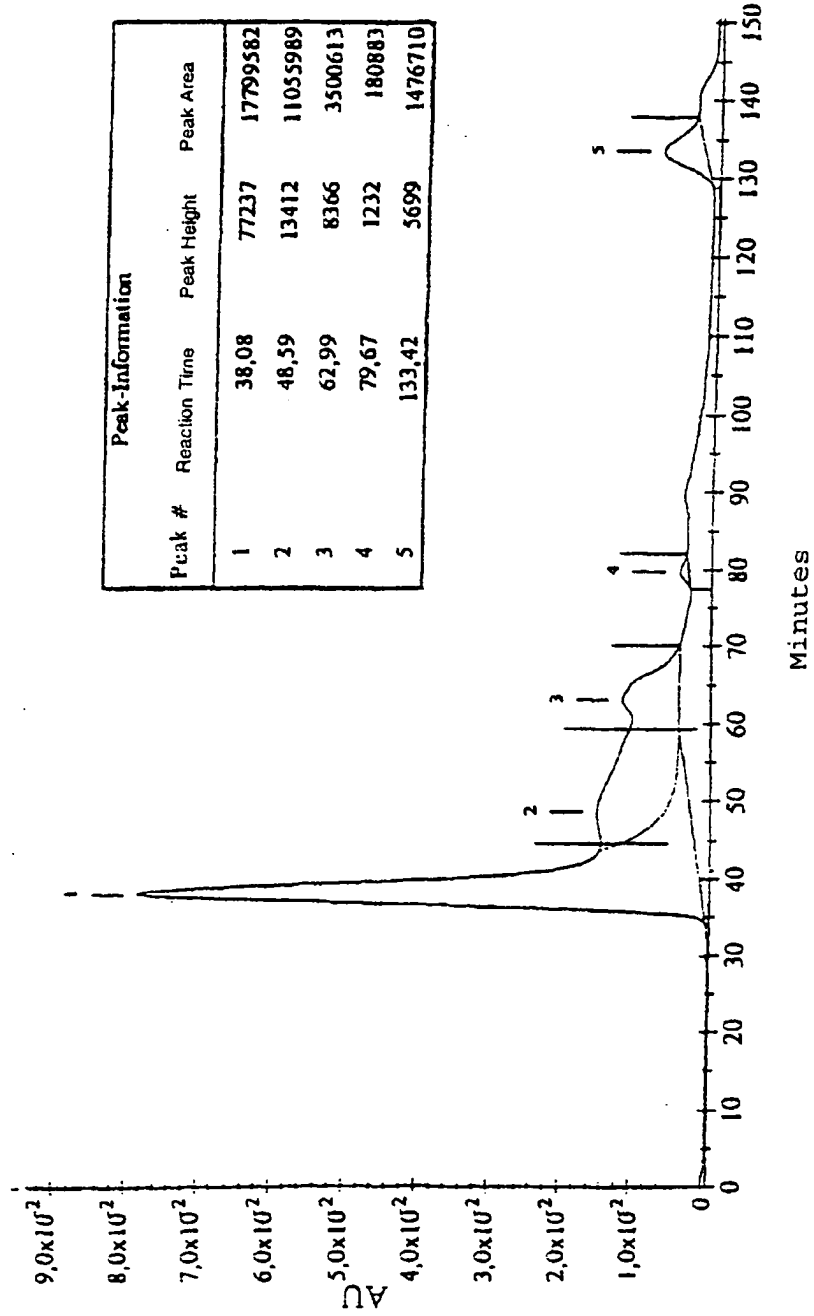


Fig. 4: GPC chromatogram of coupling V ox-HES 130 kD to HSA  
(after complete reaction)

5/12

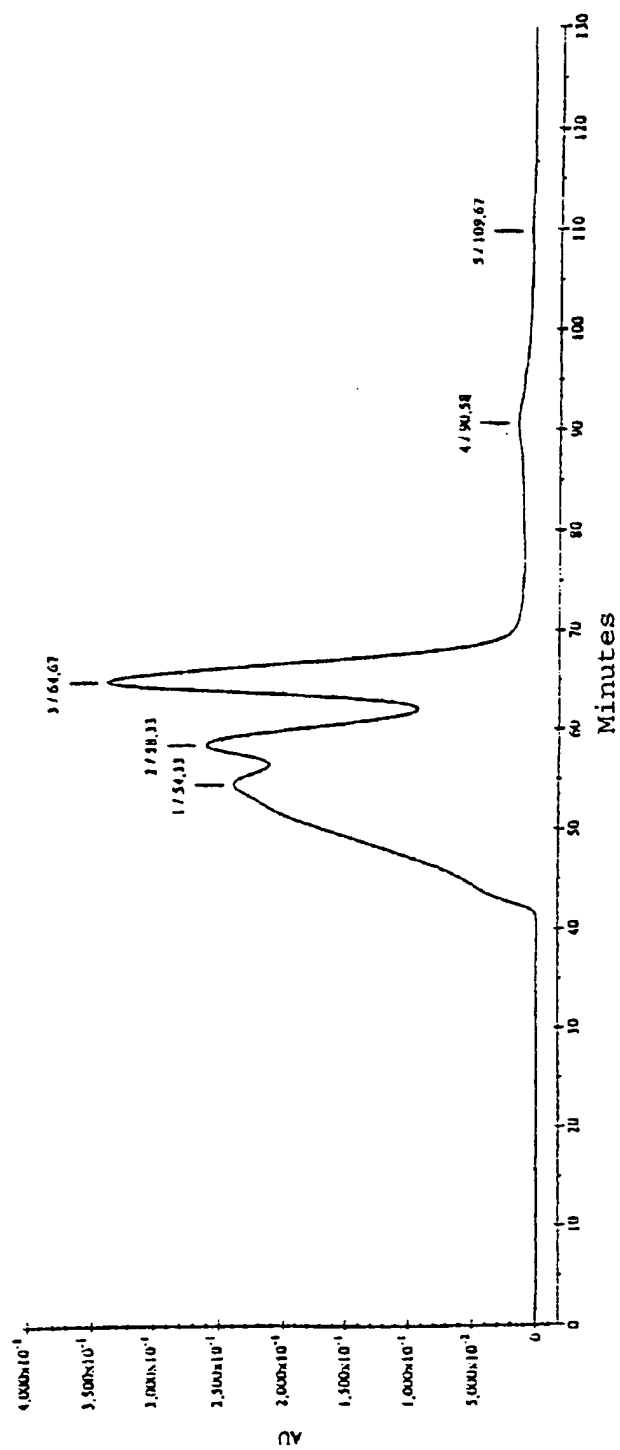


Fig. 5a: GPC analysis of the reaction kinetics of coupling V ox-HES 10 kD to HSA after 2 hours

6/12

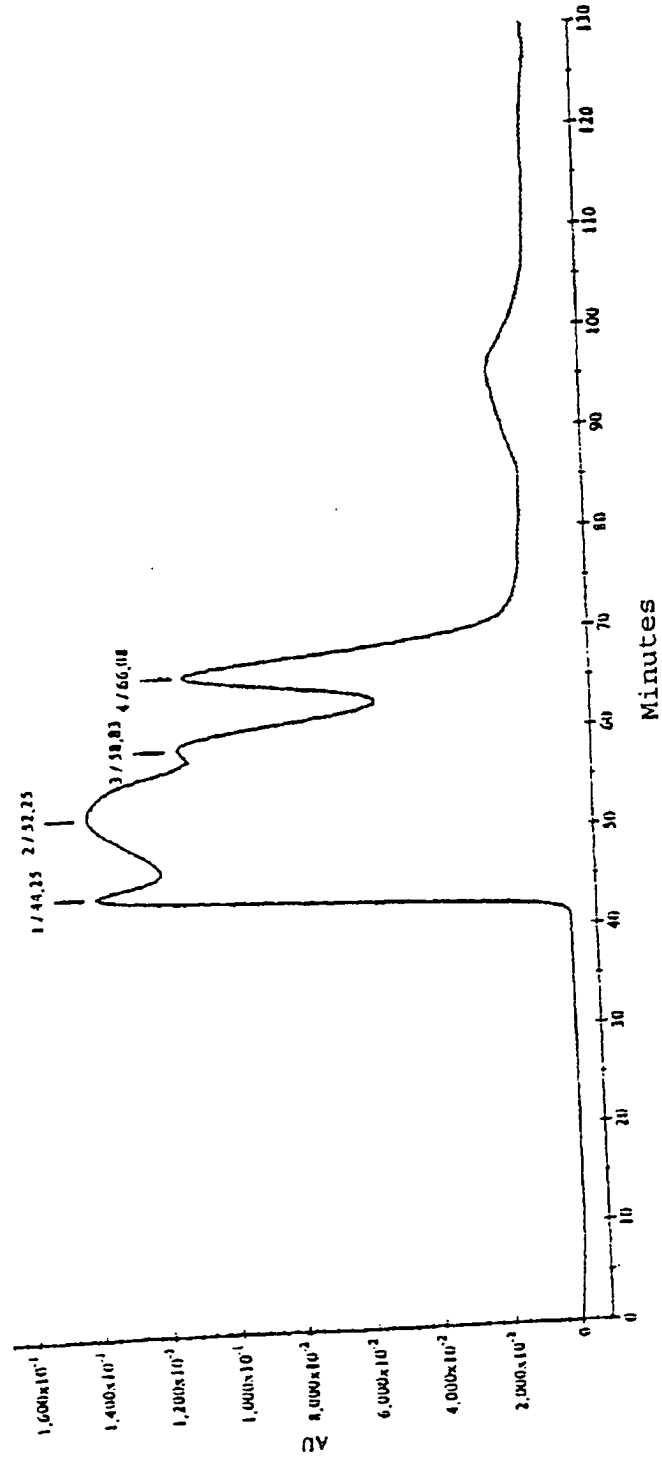


Fig. 5b: GPC analysis of the reaction kinetics of coupling V ox-HES 10 kD to HSA after 16 hours

7/12

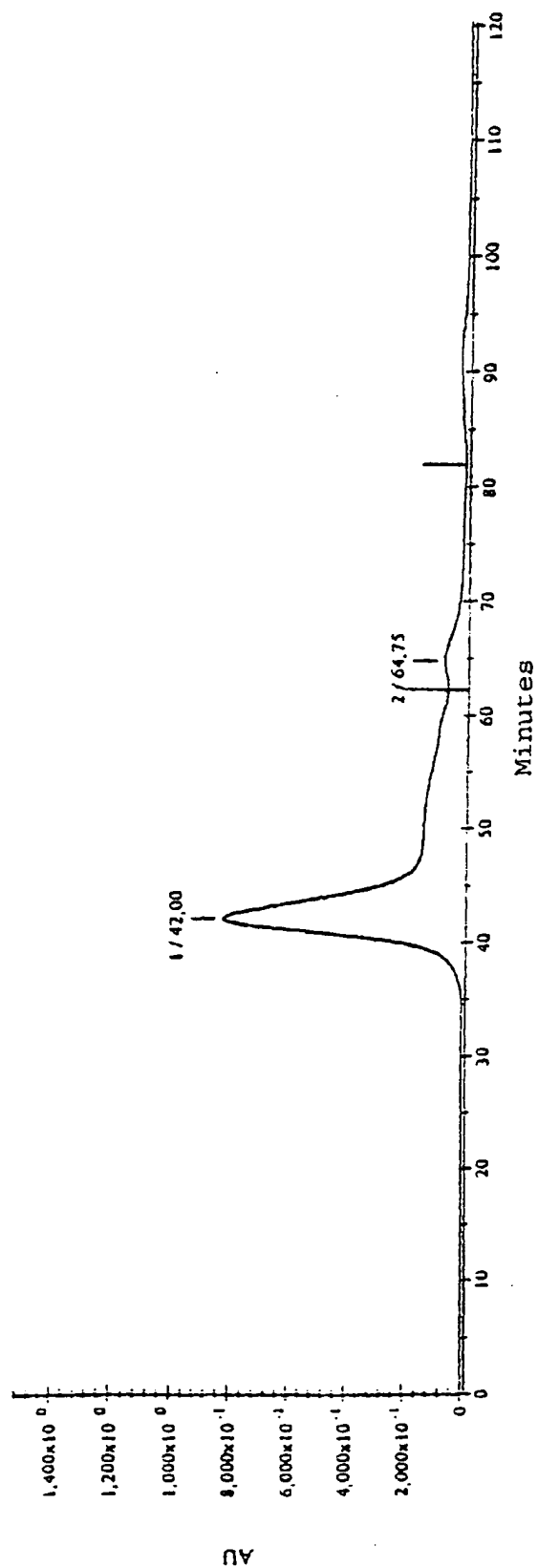


Fig. 6:

GPC analysis of the reaction mixture after 24 hours  
incubation of coupling VII ox-HES 130 kD to HSA



8/12

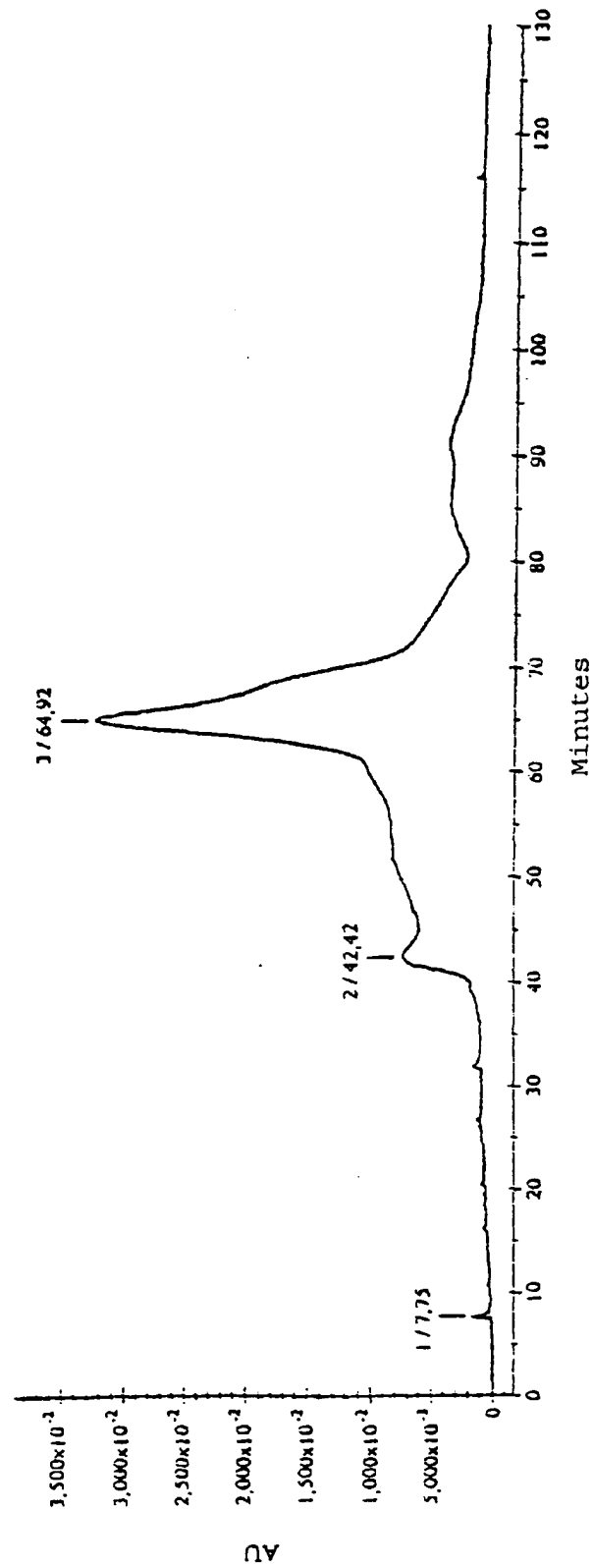
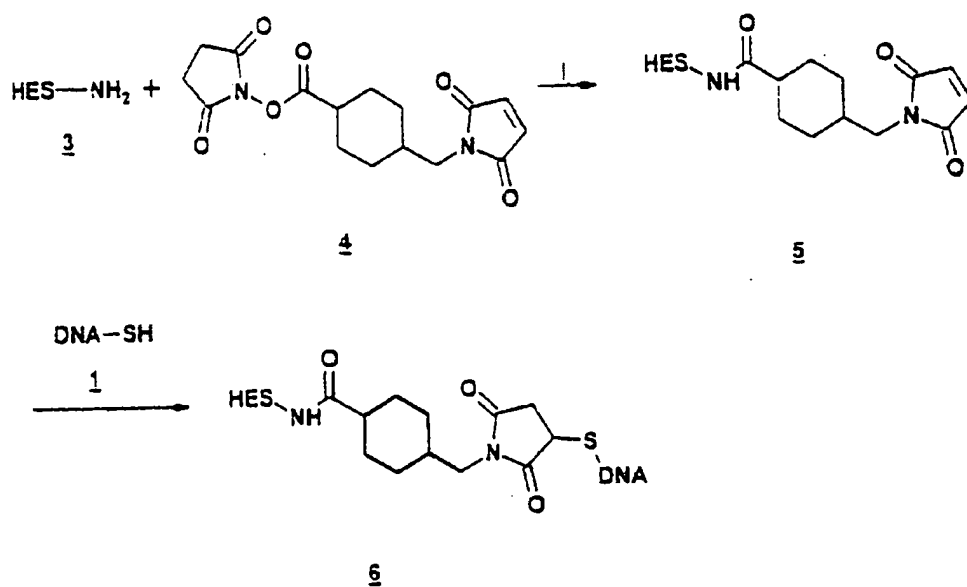


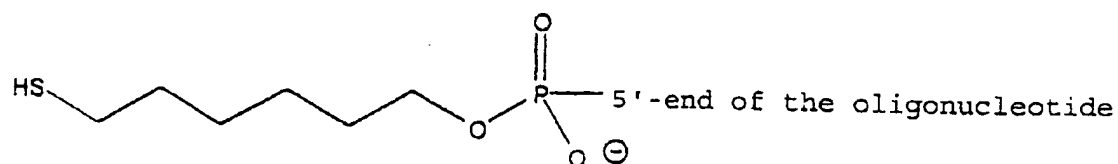
Fig. 7: GPC chromatogram of coupling V HES 130 kD to HSA and hydroboration reduction

11/12

Schematic representation of the coupling reaction between amine HES, SMCC and thio-DNA



Structure of thio-modification of the DNA



Structure of SMCC

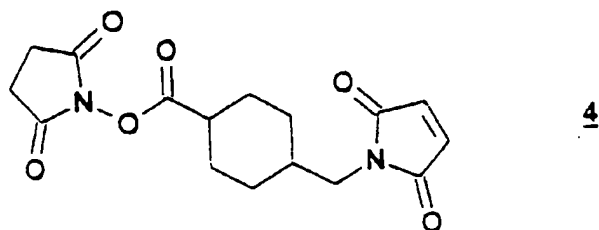


Fig. 10